the ground that "it is not clear from the specification to what characteristics of an animal this phrase refers, nor is it clear how one would judge such a characteristic."

It is respectfully submitted that the term carcass quality is well-known to those of ordinary skill in the art. The Examiner's attention is kindly invited to US Patent No. 6,248,939 B1 which issued to Leto et al. on June 19, 2001 (a copy of which is hereby included for the Examiner's convenience). This reference accompanied the previously submitted Information Disclosure Statements.

Attention is kindly invited to column 4 at lines 25-60 it is stated that to "have utility in animal feed ration as a means of improving carcass quality and subsequently improving the human diet, high oleic corn must be capably of supplying enough oleic acid in the diet to raise the oleic acid level in the meat. . . ." In other words, carcass quality refers to the composition (proportions of lean, fat and bone) of the meat. Attention in further invited to Example 3 which starts in column 16 at line 13. It is stated in column 16 at lines 17-20 that by "replacing some or all of the supplemental animal fat in a feed ratio with the oil present in high oil, high oleic corn, it will be possible to produce meat products having less saturated fats. . . ." Evaluation of carcass quality is described in column 17 at lines 33-36: the "effect of the treatments on carcass quality can be evaluated by measuring average carcass weight, average back fat, average percent lean yield, and average actual yield. . . ."

Accordingly, it is respectfully submitted that the term "carcass quality" is quite clear to one of ordinary skill in the art.

With respect to the term "carcass quality improving amount", it is respectfully submitted that this term is quite clear to one of ordinary skill in the art as demonstrated by the above quote from the '939 patent, column 4 at lines 25-60, wherein it is stated that to "have utility in animal feed ration as a means of improving carcass quality and subsequently improving the human diet, high oleic corn must be capably of supplying enough oleic acid in the diet to raise the oleic acid level in the meat. . . ." Thus, one skilled in the art would be able to determine the appropriate amount without engaging in undue experimentation. It is respectfully submitted that the metes and bounds of the claim are clear.

With respect to the language "feeding the animal", this is intended to be an active process step.

The term "reverse complement" is what the Examiner refers to as a complement on page 4 of the Office Action. It is described in the specification on page 16 at lines 29-34 with respect to "antisense RNA".

The term "shrunken 1 intron/exon" is described on page 16 at lines 3-7 at "a region of the shrunken 1 gene from corn. The **particular intron/exon used in the present** invention is derived from a non-coding region ("exon 1/intron 1") of the shrunken 1 gene **and is identical to the sequence in GenBank accession #C02382 from nucleotides 1138 through 2220**. . . ."

(Emphasis added.) It is respectfully submitted that it is quite clear what region is referred to by the claim.

The term "functionally equivalent subfragment" is referring to the nucleic acid fragment encoding a corn delta-9 stearoyl ACP desaturase.

The term "isolated nucleic acid fragment comprising a corn oleosin promoter" is referring to the promoter whether it is full length or partial.

Claims 172-174, sections (ii) and (iv), claim 175(b) and claim 176 have been rejected as being indefinite over the language "corn oleosin promoter hybridizes to the." It is respectfully submitted that when these sections are read, as a whole, it is clear that it is the isolated nucleic acid fragment (comprising the promoter sequence) which hybridizes.

Regarding claims 172 and 173, section (ii), (iii) and (iv), claim 175 and 176, it is respectfully submitted that these claims are clear with respect to what is operably linked to suitable regulatory sequences. It is stated on page 16 of the specification, starting at line 36 through line 3 of page 17 that the term "operably linked" "refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence, i.e., that the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation."

Regarding claim 175, the phrase "the corn grain" does not appear in line 2 of this claim. It simply says ". . .animal feed derived from the processing of corn grain. . . ." However, the article "a" has been inserted before corn grain to address this perceived problem. Claim 175 has also been amended to recite that the oil is comprised of not less than 60% oleic acid of the total oil content of the seed.

Claims 172-176 were rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. It is alleged on pages 5-6 of the Office that the "claims are broadly drawn in that they encompass methods for improving the carcass quality of any animal, and they do not specifically indicate the amount of feed necessary to effect the goal of improving carcass quality. Further, as discussed above, neither the claims nor the specification offer guidance as to who to measure carcass quality or what aspect of carcass quality would be improved by the consumption of the animal feed derived from the plants described in the claims. . ."

Carcass quality refers to the factors that influence the processing capabilities of both the lean and fat tissues, as well as the consumer acceptability and palatability of both fresh and processed meat products (AMSA (1971) "Meat Evaluation Handbook" American Meat Science Association Savoy, IL). Quality can be quantified using a series of laboratory

measures to evaluate weight, purge (amount of free liquid in retail packaging), appearance, water holding capacity, light reflectance, color, pH, moisture, fat, protein, cooking loss, instrumental tenderness; as well as sensory characteristics such as juiciness, tenderness, chewiness, meat flavor, off flavor and shelf life (Prusa and Fedler (1996) Proceedings: Roche Animal Nutrition and Health Veterinary Education Seminar; Meat and Livestock Commission (1996) "Blueprint for Quality British Pork", Meat Technology Transfer Group, Milton Keynes, UK). Carcass quality can also be defined in terms of the quantity and quality of specific carcass components, physical and morphological attributes, biochemical properties, microbiological and hygienic state and human nutritive properties. For example, Wood (Wood et al. (1984) "Fat in Animal Nutrition" pp 407 (Wiseman Ed) Butterworths, London) describes the important influence of the quantity and quality of carcass fat on firmness, appearance and flavor of meat from cattle, sheep and pigs; while Moran (Moran (1996) *Anim Feed Sci Tech 58*:91-99) describes opportunities to alter specific meat attributes to enhance the human health aspects of meat.

In addition the Examiner's attention is kindly invited US 6,248,939 B1 which is discussed above. Example 3 which is set forth in columns 16 and 17 discusses the use of high oil, high oleic corn in animal feed ration as a means of improving quality meat. It is stated in column 17 at lines 34-36 that the "effect of the treatments on carcass quality can be evaluated by measuring average carcass weight, average back fat, average percent lean yield and average actual yield." Thus, one of ordinary skill in the art would know how to measure/assess carcass quality. Such knowledge existed prior to the filing date of the instant application. Determination of the amount of feed can readily be assessed by one skilled in the art without engaging in undue experimentation.

The law is well settled that the specification need not teach or disclose in detail that which is well known in the art. Genentech, Inc. v. Novo Nordisk A/S, 42 USPQ2d1001, , 1005 (Fed. Cir.1997); In re Meyers, 162 USPQ 668 (CCPA 1969).

The references cited on page 6 of the Office Action seem to be inapposite in that they concern replacing maize with barley. The instant invention concerns replacing standard maize with corn grain obtained from a corn plant or plant part comprising any of the chimeric genes recited in the claims. Thus, the present invention concerns altering oil profile in corn using chimeric genes comprising nucleic acid fragments described in the application and suitable regulatory sequences to create transgenic corn plants having altered lipid profiles and this altered corn can then be used as an animal feed ration to improve carcass quality.

Applicant respectfully submits that in view of the above discussion and reference, undue experimentation would not be required to practice the claimed invention.

It is alleged on page 8 of the Office Action that the "specification teaches plants in which sense and anti-sense nucleic acids encoding corn delta-9 stearoyl ACP desaturase are introduced into plants, and in both instances the resulting plant displayed high saturate fatty

acid composition. The mechanism by which this occurs is unclear, and therefore, it is not possible to predict the effect that adding other nucleic acids to the plants would have on the plant. . . . Due to the lack of guidance in the specification, the high level of unpredictability with regard to which nucleic acids would be useful for producing such plants, undue experimentation would be required to produce animal feed from plants as broadly claimed."

The law is well settled that it is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works. See, Newman v. Quigg, 11 USPQ2d 1340, 1345 (Fed. Cir. 1989).

The Examiner's attention is kindly invited to Example 8 in the specification which describes (1) transgenic corn with high saturate fatty acid composition in the grain, (2) transgenic corn with a high oleic acid content in the grain and (3) transgenic corn with high levels of saturated and oleic acid in kernels. The specification and examples show one of ordinary skill in the art how the practice the claimed invention.

Claims 172-176 were rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor at the time the application was filed, has possession of the claimed invention.

The term "functionally equivalent subfragment" is described in the specification on page 13 at lines 29-37 and page 14 discusses the % identity language. Thus, contrary to the allegation on page 10 of the Office, it is respectfully submitted that there is written description in the specification for the foregoing language.

In addition, the examples clearly demonstrate conception of the invention. There is no magical relationship between the number of representative examples and the breadth of the claims. The number and variety of examples are irrelevant if the disclosure is "enabling" and sets forth the "best mode contemplated." In re Borkowski, 164 USPQ 642 (CCPA 1970).

It is contended on page 10 of the Office Action that ". . . the mechanism by which the introduced nucleic acids act in plants is unkown, and therefore 'the function' of the nucleic acids in the plants is unknown." As was indicated above, and is reiterated here, the law is well settled that it is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works. See, Newman v. Quigg, 11 USPQ2d 1340, 1345 (Fed. Cir. 1989).

Accordingly, in view of the above discussion, withdrawal of the rejection of the claims under 35 USC §112, first and second paragraphs, is respectfully requested.

A petition for a three (3) month extension of time accompanies this response along with the Version with Markings To Show Changes Made and copies of any references noted in the response.

It is respectfully submitted that the claims are now in form for allowance which allowance is respectfully requested.

Please charge any fees associated with the filing of this response to Deposit Account No. 04-1928 (E. I. du Pont de Nemours and Company). If the fee is insufficient or incorrect, please charge or credit the balance to the above-identified deposit account.

Respectfully submitted,

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Dated: November 5, 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In showing the changes, deleted material is shown Bracketed, and inserted material is shown as underlined.

In the Abstract

Kindly delete the Title and Abstract appearing on page 61 in their entirety and replace it with the following:

--TITLE

A METHOD FOR IMPROVING THE CARCASS QUALITY OF AN ANIMAL

ABSTRACT

A method for improving the carcass quality of an animal is described. This method involves the preparation and use of nucleic acid fragments comprising all or substantially all of a corn oleosin promoter, a stearoyl-ACP desaturase and a delta-12 desaturase which can be used individually or in combination to modify the lipid profile of corn are described. Chimeric genes incorporating such nucleic acid fragments and suitable regulatory sequences can be used to create transgenic corn plants having altered lipid profiles are also described. --.

In the Specification

Page 1, lines 1 and 2, delete "TITLE GENES FOR DESATURASES TO ALTER LIPID PROFILES IN CORN" and replace it with the following:

--TITLE

A METHOD FOR IMPROVING THE CARCASS QUALITY OF AN ANIMAL--.

Page 14, lines 9-36 has been amended as follows:

--Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under moderately stringent conditions (for example, 0.5 X SSC, 0.1% SDS, 60° C) with the sequences exemplified herein, or to any portion of the nucleotide sequences reported herein and which are functionally equivalent to the promoter of the invention. Preferred substantially similar nucleic acid sequences encompassed by this invention are those sequences that are 80% identical to the nucleic acid fragments reported herein or which are 80% identical to any portion of the nucleotide sequences reported herein. More preferred are nucleic acid fragments which are 90% identical to the nucleic acid sequences reported herein, or which are 90% identical to any portion of the nucleotide sequences reported herein. Most preferred are nucleic acid fragments which are 95% identical to the nucleic acid sequences

reported herein, or which are 95% identical to any portion of the nucleotide sequences reported herein. Sequence alignments and percent similarity calculations may be determined using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences are performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identiy of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are GAP PENALTY=10, GAP LENGTH PENALTY=10, KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F., et al., (1993) *J. Mol. Biol. 215*:403-410) and Gapped Blast (Altschul, S. F. et al., (1997) *Nucleic Acids Res. 25*:3389-3402)[; see also www.ncbi.nlm.nih.gov/BLAST/)].

In the Claims

175. (once amended) A method of improving the carcass quality of an animal by feeding the animal a carcass quality improving amount of animal feed derived from the processing of a corn grain obtained from a corn plant or plant part which comprises a chimeric gene comprising (a) an isolated nucleic acid fragment encoding a corn delta-12 desaturase wherein said fragment has a nucleic acid sequence identity of at least 80% based on the Clustal method of alignment when compared to a nucleic acid as set forth in SEQ ID NOS: 1, 58 or 59, or a functionally equivalent subfragment thereof, or the reverse complement of either the fragment or subfragment, (b) an isolated nucleic acid fragment comprising a corn oleosin promoter wherein said promoter can be full length or partial and said promoter: (1) comprises a nucleotide sequence having a sequence identity of at least 80% based on the Clustal method of alignment when compared to the nucleotide sequence in any of SEQ ID NOS:19 or 38-49 or (2) the isolated nucleic acid fragment comprising a full length or partial corn oleosin promoter hybridizes to the nucleotide sequence set forth in SEQ ID NOS: 19 or 38-49 under moderately stringent conditions, operably linked to suitable regulatory sequences, and (c) a shrunken 1 intron/exon, operably linked to suitable regulatory sequences;

wherein expresssion of the chimeric gene results in an altered corn oleic acid phenotype, and

further wherein the corn grain has an oil content in the range from about 6% to about 10% on a dry matter basis and further wherein said oil is comprised of not less than [about] 60% oleic acid of the total oil content of the seed.

US006248939B1

(12) **United States Patent**
Leto et al.

(10) **Patent No.: US 6,248,939 B1**
(45) **Date of Patent: Jun. 19, 2001**

(54) **CORN PLANTS AND PRODUCTS WITH IMPROVED OIL COMPOSITION**

(75) Inventors: **Kenneth Joseph Leto**, West Des Moines, IA (US); **James Francis Ulrich**, Aurora, IL (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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§ 102(e) Date: **Aug. 26, 1996**

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PCT Pub. Date: **Aug. 24, 1995**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/196,622, filed on Feb. 15, 1994, now abandoned.

(51) **Int. Cl.**[7] **A01H 5/00**; A01H 4/00

(52) **U.S. Cl.** **800/320.1**; 800/298; 800/275

(58) **Field of Search** ..... 800/200, 205, 800/230, 235, 250, DIG. 56, 320.1, 298, 275; 47/58, DIG. 1; 435/172.3, 172.1, 424, 430, 430.1

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(57) **ABSTRACT**

This invention relates to corn (*Zea mays* L.) seed and grain having a significantly higher oleic acid content than conventional corn by virtue of heritable genes for increased oil and oleic acid content and/or lowered levels of linoleic acid. The present invention also relates to the production of high oil, high oleic grain, its oil, its progeny and its use.

16 Claims, No Drawings

CORN PLANTS AND PRODUCTS WITH IMPROVED OIL COMPOSITION

This application is a 371 of PCT/US95/02076 filed Feb. 15, 1995, which is a Continuation-in-part of Ser. No. 08/196,622 filed Feb. 15, 1994 now abandoned.

FIELD OF THE INVENTION

This invention relates to corn (*Zea mays* L.) seed and grain having a significantly higher oleic acid content by virtue of heritable genes for increased oil and oleic acid content and/or lowered levels of linoleic acid. The present invention also relates to the production of high oil, high oleic grain, plants and plant parts grown from such grain and uses of such improved grain.

TECHNICAL BACKGROUND OF THE INVENTION

Corn oil is comprised primarily of even-numbered carbon chain fatty acids. The distribution of fatty acids in typical corn oil is approximately 12% palmitic acid (16:0), 2% stearic acid (18:0), 25% oleic acid (18:1), 60% linoleic acid (18:2), and 1% linolenic acid (18:3). Palmitic and stearic acids are referred to as saturated fatty acids because their carbon chains contains only single bonds and the carbon chain is "saturated" with hydrogen atoms. Oleic, linoleic, and linolenic acids contain one, two, and three double bonds respectively, and are referred to as unsaturated fatty acids. Fatty acids in corn oil nearly always occur esterified to the hydroxyl groups of glycerol, thus forming triglycerides. Approximately 99% of refined corn oil is made up of triglycerides; Corn Oil, Corn Refiners Association, Inc., 1001 Connecticut Ave., NW., Washington, DC 20036, 1986, 24 pp.

When exposed to air, unsaturated fatty acids are subject to oxidation which causes the oil to have a rancid odor. Oxidation is accelerated by high temperatures, such as in frying conditions. The rate of oxidation is proportional to the number of double bonds in the fatty acids. Thus, linoleic acid with two double bonds is more unstable than oleic acid which has only one double bond. Oxidation reduces the shelf life of products containing corn oil because of the oil's high proportion of linoleic acid. Corn oil and products containing corn oil are often packaged under nitrogen in special packaging materials such as plastic or laminated foil, or are stored under refrigeration to extend their shelf life. These extra measures to reduce oxidation and subsequent rancidity add considerable cost to products containing corn oil.

Another measure to reduce the effects of oxidation on corn oil is to chemically hydrogenate the oil. This commercially important process by which hydrogen is added to double bonds of unsaturated fatty acids changes the physical properties of the oil and extends the shelf life of products containing corn oil. Hydrogenated vegetable oils are used to make margarine, salad dressings, cooking oils, and shortenings, for example. Approximately half a billion pounds, or roughly 40–50% of corn oil produced in the U.S. is used for cooking and for salad oils; Fitch, B., JAOCS, 1985, Vol. 62, no. 11, pp. 1524–31. Production of a more stable oil by genetic means would clearly have value by reducing or eliminating the time and input costs of chemical hydrogenation.

In addition to the economic factors associated with chemical hydrogenation of corn oil, there are human health factors that favor the production of a natural high oleic oil. During the hydrogenation process, double bonds in fatty acids are completely hydrogenated or are converted from the cis configuration to the trans configuration. Cis double bonds cause a fatty acid molecule to bend, which impairs crystallization and keeps the oil liquid at room temperature. During hydrogenation, cis bonds are straightened into the trans configuration, causing the oil to harden at room temperature. Recent studies on the effect of dietary trans fatty acids on cholesterol levels show that the trans isomer of oleic acid raises blood cholesterol level at least as much as saturated fatty acids, which have been know for some time to raise cholesterol in humans; Mensink, R. P. and B. K. Katan, N. Engl. J. Med., 1990, 323:439–45. Furthermore, the studies show that the undesirable low density lipoprotein level increases and the desirable high density lipoprotein level decreases in response to diets high in trans fatty acids. Large amounts of trans fatty acids are found in margarines, shortenings, and oils used for frying; the most abundant trans fatty acid in the human diet is the trans isomer of oleic acid, elaidic acid. A natural high oleic corn oil, which does not contain elaidic acid, will benefit consumers in general, and will particularly benefit those people who control their cholesterol level through their diet.

The human diet could also be improved by reducing saturated fat intake. Much of the saturated fat in the human diet comes from meat products. Poultry and swine diets often contain animal fat, which is high in saturated fatty acids, as an energy source. Non ruminant animals such as these are very susceptible to tissue fatty acid alteration through dietary modification; M. F. Miller, et al., J. Anim. Sci., 1990, 68:1624–31. A large portion of animal feed rations is made up of corn, which typically contains only about 4% oil. By replacing some or all of the supplemental animal fat in a feed ration with the oil present in high oil corn varieties, which contain up to 10% oil, it will be possible to produce meat products having less saturated fats. Feeding trials in which swine were fed diets high in oleic acid show that the amount of oleic acid deposited in adipose tissue can be raised substantially without adversely influencing the quality of the meat; M. F. Miller, et al., supra; L. C. St. John, et al., J. Anim. Sci., 1987, 64:1441–47. The degree of saturation of the fatty acids comprising an oil determines whether it is liquid or solid. In these studies, the animal diets high in oleic acid led to meat quality that was acceptable to the meat processing industry because of the low level of polyunsaturated fatty acids. Therefore, it can be extended that a feed ration containing high oleic, high oil corn would be preferable to one containing high oil corn which contains a high level of linoleic acid. Consumption of monounsaturated fatty acids decreases the LDL level without affecting the HDL level; Mattson, F. R., and S. M. Grundy, J. Lipid Res., 1985, 26:194. The HDL portion is responsible for removal of cholesterol from the body; L. C. St. John, supra. Processed meats produced from animals fed diets containing high oil, high oleic corn will be more healthful in the human diet.

The corn kernel is a product of double fertilization; Kiesselbach, T. A., 1980, The Structure and Reproduction of Corn, University of Nebraska Press. This means that both the diploid embryo (giving rise to the germ and seedling) and the triploid endosperm (the nutritive structure surrounding the germ) contain genes transmitted from both the male and female parents. Nonetheless, the genes affecting grain composition and quality are similar enough in most field corn inbreds that crossing any given female with a large variety of male plants does not result in dramatic changes in the compositional or quality characteristics of the resulting seed or grain. Likewise, planting different field corn hybrids

within pollinating proximity to each other will not, in most cases, substantially affect the quality of the grain harvested on each type.

In contrast, a minority of commercial corn inbreds or hybrids do contain genes which substantially modify grain quality. These hybrids, include those containing the waxy gene. Such waxy gene hybrids must be isolated from normal, non-waxy corn inbreds or hybrids in order to recover waxy seed or grain. If a non-waxy pollen grain (as found in most field corn inbreds and hybrids) pollinates an ovule borne on a waxy inbred or hybrid, the resulting kernel will be non-waxy, even though adjacent kernels on the same ear, pollinated by waxy pollen, will remain waxy. This immediate effect of pollen genotype on kernel characteristics is termed "xenia", and the hybrid nature of such kernels is recognizable by particular phenotypic characteristics (color, shape, size, etc.) owing to the direct influence exerted by the genotype of the pollen; Rieger, R., A. Michaelis and M. M. Green, 1968, A Glossary of Genetics and Cytogenetics, Springer-Verlag, New York. This immediate effect of pollen genotype on grain quality has been observed with pollen obtained from high-oil corn plants; Alexander, D. E. and R. J. Lambert, 1968, Relationship of Kernel Oil Content to Yield in Maize Crop Science 8:272–274.

Production of oleic acid in corn is under genetic control, although the mode of inheritance is only partially understood. Oil production in the kernel occurs primarily in the germ. Fatty acid biosynthesis is regulated by a multi-step biochemical pathway whereby the saturated fatty acids, palmitic and stearic, are synthesized and subsequently dehydrogenated to oleic, linoleic, and linolenic acids; Lipid Metabolism, In: Introduction to Plant Biochemistry, 2nd Ed., 1983, Pergamon Press, Goodwin and Mercer, Eds., pp 273–327. A single gene locus, designated In, was reported to be responsible for regulating the levels of oleic and linoleic acids in corn; Ponelelt, C. G., and D. E. Alexander, Science, 1965, 147:1585–86. Subsequent studies show that the mode of inheritance of oleic acid is more complicated than first thought. At least two loci have been shown to regulate the oleic acid level; de la Roche et al., Crop Sci., 1971, 11:856–59. In a study involving eight different reciprocal crosses and their parental inbred lines, it was concluded that inheritance of increased oleic content in corn can result from dominant, partially dominant, and even recessive gene action; Jellum, M. D., J. Hered., 1966, 57:243–44. Only one report has been found in which the inheritance of oleic acid in a high oil corn line, IHO, is discussed; de la Roche, et al., supra. The report states that the quality of corn oil increases as the linoleic acid content increases. The data are presented in terms of the linoleic acid content, which for IHO is reported to be approximately 47% of the oil fraction. From our studies of thousands of samples, there is an inverse relationship between oleic acid and linoleic acid content. A line that is 47% linoleic acid would contain 35–40% oleic acid, which is substantially less than the oleic content in the present invention. Also, IHO is not an agronomically acceptable line and would not be used in commercial production; Glover, D. V., and E. T. Mertz, Corn, In Nutritional Quality of Cereal Grains: Genetic and Agronomic Improvement, Agronomy Monograph no. 28, Copyright 1987, ASA-CSSA-SSSA, 677 South Segoe Road, Madison, Wis. 53711, USA, Chapter 7, pp. 183–336; Fitch, B., JAOCS, 1985, Vol. 62, no. 11, pp. 1524–31.

A survey of plant introductions for fatty acid profile shows that greater genetic diversity exists in corn of foreign origin than exists in U.S. corn; Jellum, M. D., 1970, J. Agr. Food Chem., 18:3, pp. 365–70. Oleic acid content ranged from 14 to 64% in the plant introductions screened, which represented germplasm from over 50 foreign countries. Plant introductions are a valuable source of genetic diversity for many traits, including oleic acid content. However, breeding genes from plant introductions of foreign origin into elite U.S. adapted inbred lines is a costly process requiring three to six years.

A breeding strategy know as recurrent selection has been suggested as a means of increasing the oleic acid level in corn; Ponelelt, C. G., and L. F. Bauman, Crop Sci., 1970, 10:338–41. This breeding method was applied to maize plant introductions and is the basis for a patent application for high oleic corn products and methods for their production; PCT/US91/04626. To have commercial utility, the value of a trait, such as high oleic oil, must be worth more than the costs associated with production, storage, and shipment of the grain. A bushel of shelled corn, which weighs approximately 56 pounds, can yield approximately two pounds of oil when milled. Because of the small amount of oil normally found in corn, the added value of an improved oil, such as high oleic oil, is unlikely to be sufficient to pay for the production and identity preservation costs, unless substantially greater oil is produced as in newly developed high oil corn varieties.

To have utility in an animal feed ration as a means of improving carcass quality and subsequently improving the human diet, high oleic corn must be capable of supplying enough oleic acid in the diet to raise the oleic acid level in the meat. Corn is included in animal feed as the main source of energy, the majority of which comes from the high starch content of corn, and other sources of energy such as animal fat, vegetable fat, or animal-vegetable fat blends are commonly added to increase the energy density of feed rations. For example, the amount of corn oil included in the corn fraction of a typical commercial poultry feed ration is about 2.5% in a ration that contains 65–70% corn. To increase the energy density of feed rations, highly saturated animal fat or animal-vegetable fat blends are added at approximately 5 to 8% of the diet. High oil corn with an energy content which is significantly higher than that of normal corn can reduce or totally eliminate the use of or need for added fat when used in a typical poultry ration.

A typical chicken broiler corn-soybean meal diet supplemented with an animal-vegetable fat blend contains approximately 1.937% oleic acid. Increasing the oleic acid content of the oil contained in corn used in a feed ration from the 25% found in normal corn to 60% (also in a normal or low oil variety) increases the oleic acid in the feed ration to 2.733%. Increasing the oleic acid content from 25% to 60% of the oil present in high oil corn grain that contains 8–10% oil increases the oleic acid content of the feed ration to 4.266%. These increases represent a 30% increase in oleic acid content when normal corn is used in the feed ration, and a 120% increase when high oil corn varieties are used. High oil corn can reduce or totally eliminate the need for added fat when used in a typical poultry ration, suggesting that modifications to the fatty acid profile of corn oil need to be made in a high oil corn variety to have utility in improving carcass quality. The high oleic corn lines described in the aforementioned patent application are not high oil corn lines.

Most cereal crops are handled as commodities, and many of the industrial and animal feed requirements for these crops can be met by common varieties which are widely grown and produced in volume. However, there exists at present a growing market for crops with special end-use properties which are not met by grain of standard composition. Most commonly, specialty maize is differentiated

from "normal" maize, also known as field corn, by altered endosperm properties, such as an overall change in the degree of starch branching, as in waxy or high amylose maize, an increased accumulation of sugars as in sweet corn, or an alteration in the degree of endosperm hardness as in food grade maize or popcorn; Glover, D. V. and E. T. Mertz, 1987, Corn. In: Nutritional Quality of Cereal Grains; Genetic and Agronomic Improvement, R. A. Olson and K. J. Frey, eds. American Society of Agronomy, Madison, Wis., pp. 183–336; Rooney, L. W. and S. O. Serna-Saldivar, 1987, Food Uses of Whole Corn and Dry-Milled Fractions, In: Corn:Chemistry and Technology, S. A. Watson and P. E. Ramstead, eds. American Association of Cereal Chemists, Inc., St. Paul, Minn., pp. 399–429. "Specialty" crops are typically grown under contract for specific end users who place value on starch quality or other specific quality attributes. A specialty crop such as waxy maize is more valuable as a raw material to the starch industry than is normal or commodity grade maize, and thus is referred to as a value added crop. Currently the market size and added value of waxy maize is such that approximately 150,000 acres are grown in the United States. Farmers are paid a premium for growing specialty crops such as waxy maize because it is more valuable than normal maize and must not be mixed with normal maize. Because of the desire of many humans to eat a healthier diet and the documented effects of oleic acid on reducing cholesterol, the present invention will have greater value than normal corn. The current invention offers farmers the opportunity to grow a higher value crop than normal maize.

Oil is obtained from plants by a milling process. Corn oil is extracted from kernels through the use of a either a wet or dry milling process. Wet milling is a multi-step process involving steeping and grinding of the kernels and separation of the starch, protein, oil, and fiber fractions. A review of the maize wet milling process is given by S. R. Eckhoff in the Proceedings of the Fourth Corn Utilization Conference, Jun. 24–26, 1992, St. Louis, Mo., printed by the National Corn Growers Association, CIBA-GEIGY Seed Division and the United States Department of Agriculture. Dry milling is a process by which the germ and hull of the corn kernel are separated from the endosperm by the controlled addition of water to the grain and subsequent passage through a degerming mill and a series of rollers and sieves. The U.S. dry milling industry produces approximately 50 million pounds of crude corn oil per year, and the wet milling industry produces over one billion pounds of crude corn oil; Fitch, 1985, supra. The present invention offers the wet and dry milling industries the opportunity to process and sell a higher value oil than normal corn oil.

SUMMARY OF THE INVENTION

Applicants have discovered a corn plant capable of producing grain having a ten fold increase in oleic acid content over normal corn by breeding a high oil corn variety with a corn variety that carries a chemically mutated gene that confers high oleic acid content. Specifically, a corn plant has been bred to produce grain having 17 to 20% oil, with about 60% of the oil being oleic acid. Plants of this type can be used to pollinate high yielding, commercially acceptable hybrids that are male sterile, which have high oleic acid producing characteristics, thus producing grain having a five fold increase in oleic acid content over normal corn. By using this method and pollinator plants of this type, the timeline for commercial production of corn having elevated oleic levels is greatly accelerated.

This invention consists of corn grain produced by planting in close proximity, preferably in a field, corn plants of an agronomically elite high-yielding female parent, having high oleic characteristics, and optionally having high-oil characteristics, with corn plants of a high-oil and high oleic male parent, optionally having high-yielding characteristics and/or agronomically elite characteristics. The grain may then be all harvested or harvested selectively, for example, so that kernels produced by the female plants are harvested as grain. In the planting described, the preferred high-oil, high oleic male parent plant, when self or sib pollinated, is capable of producing kernels having a total oil content ranging from 7.5% to 20% of the total seed weight, measured at zero percent moisture and an oleic acid content of not less than about 55% of the total oil content of the seed. The agronomically elite female parent, when self or sib pollinated, is capable of producing kernels having a total oil content of between about 2 percent to about 7.5 percent, preferably at least about 6%, of the total seed weight, measured at zero percent moisture, wherein the oleic acid content is not less than about 55% of the total oil content.

The grain produced has as oleic acid content of about 4% to about 7% of the total seed weight.

The present invention further comprises a corn oil, produced from the grain described above, which has 50% to 120% greater oxidative stability than that of "normal" corn oil where the oxidative stability is obtained without the addition of antioxidants. Such oil is useful in human and animal food, in cooking, and in industrial applications.

Also taught herein is a method of developing corn varieties with altered levels of fatty acid compositions, including oleic acid, comprising treatment of seeds or pollen with a chemical mutagen to produce mutant plants. Mutagens useful herein are selected from ethylmethanesulfonate and nitrosomethylurea. Also taught is a corn variety produced by such method having total oleic acid content of not less than about 55% of the total oil content of the seed, when measured at about zero percent moisture.

A further embodiment of the invention relates to the use of high oil, high oleic grain as an animal feed to improve meat quality, particularly in swine and poultry.

The present invention further comprises mutant corn lines B73OL and AEC272OL lines which bear the ATCC accession numbers 97026, 97027, from which the high oleic characteristics of the male and female plants that are crossed, as described above, are generated. The invention also describes the high oil, high oleic corn variety resulting from the cross, ASKC28 X B73OL, which bears the ATCC accession number 97042. The mutant corn lines deposited under ATCC accession numbers 97026 and 97207 were deposited under terms conforming to the Budapest Treaty on Jan. 27, 1995.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides corn plants that produce grain having a mean oleic acid content of about ten percent, or approximately ten times the oleic acid content of "normal" corn. A valuable property of this higher oleic acid type of corn is the increased oxidative stability of its oil. Use of the grain, produced by these corn plants, in feeding results in improved carcass quality of animals.

One method of enhancing the oleic acid content of corn grain comprises a planting, as described in the Summary of the Invention, involving the steps of:

(a) planting in close proximity, in a field:

(1) corn seed of a high-yielding, agronomically elite variety to obtain female (i.e., male sterile) corn

plants which have a high oleic acid characteristics, and which may or may not have an oil content greater than that of normal corn; and

(2) corn seed of high-oil, high oleic variety male corn plant, which may or may not have high yielding characteristics, and which further may or may not be nonisogenic to said female corn plants, so as to produce high-oil, high oleic corn plants capable of serving as pollinators,

(b) permitting said high-oil, high oleic corn plants to pollinate said female corn plants;

(c) harvesting the resulting corn grain on said corn plants, thereby obtaining a high yield of corn grain possessing an oil concentration not less than about 7.5% and oleic acid content intermediate between that found in kernels obtained following self-pollination of said pollinator and said female corn plants.

To facilitate cross pollination, the plants to be used as the female are rendered male sterile. This can be accomplished by physical removal of the male pollen-shedding part of the plant, by chemical treatment, or by a genetic mechanism such as cytoplasmic male sterility. In maize, the male part of the plant is the tassel which can be easily removed by hand or machine. Production of the present invention in maize requires planting male and female genotypes in adjacent rows in the field or, preferably, intermixed within the same rows. Female plants are rendered male sterile, preferably through genetic means, and are pollinated by male plants. Grain is harvested from female and male plants for subsequent oil extraction.

It has also been observed that a gene altered through chemical mutagenesis can be used to alter the oleic acid level as it confers the same increase in oleic acid as a percent of total oil in corn seeds that contain about 20% oil as in seeds that contain only about 4% oil.

A major advantage of oil obtained from grain produced in accordance with the present invention is that it possesses greater oxidative stability than normal corn oil, without employing antioxidants. When used in food and frying applications, oil obtained from the grain of the present invention will have a longer shelf life or fry life and will not develop rancid odors as quickly as normal corn oil. Oil of the present invention can replace chemically hydrogenated oil in applications where oxidative stability is desired.

For purposes of this application, unless otherwise noted, the oleic acid content or % oleic acid is the percent of the total fatty acids in the oil, which also includes, but is not limited to, palmitic acid, stearic acid, linoleic acid, and linolenic acid.

In the context of this disclosure, a number of terms shall be utilized relevant to plant breeding and oil characterization. As used herein, an "allele" is one of two or more forms of a gene that exists at a chromosome location. The term "corn" refers to any variety, cultivar or population of *Zea mays* L. The terms "commercially acceptable" or "elite" characterize a plant or variety possessing favorable traits, such as, but not limited to, high yield, good grain quality, and disease resistance. This enables its use in commercial production of seed or grain at a profit. These terms also characterize parents giving rise to such plants or varieties. "Field corn" refers to varieties or cultivars of corn grown extensively in large acreage for the production of grain and/or forage. Most field corn in the United States is also referred to as dent corn, whereas field corn produced in Europe and Argentina is more likely to be referred to as flint corn. The "germ" is the embryo of the corn kernel and contains the vast majority of the oil found in the kernel. "Grain" comprises mature corn kernels produced by commercial growers for on farm use or for sale to customers in both cases for purposes other than growing or reproducing the species. Typical customers would include livestock feeders, wet or dry millers, or animal feed formulators.

The term "heterozygous" describes a genetic condition existing when different alleles reside at corresponding loci on homologous chromosomes. A "high-oil corn kernel" is one which contains elevated levels of oil on a percent dry weight basis when compared to low-oil corn kernels. A "high-oil corn plant" is a plant which, when self pollinated, will give rise to kernels containing elevated levels of oil on a percent dry weight basis when compared to a low-oil corn plant. The term "homozygous" describes a genetic condition existing when identical alleles reside at corresponding loci on homologous chromosomes. A "hybrid" represents any offspring of a cross between two genetically unlike individuals; Rieger R., A. Michaelis and M. M. Green, 1968, A Glossary of Genetics and Cytogenetics, Springer-Verlag, New York. An "inbred" is a substantially homozygous plant or variety. The "kernel" is the corn caryopsis, consisting of a mature embryo and endosperm which are products of double fertilization. A "low-oil corn kernel" contains oil in the range of about 2.5 to 5.1 percent on a dry weight basis. A "low-oil corn plant" is one which, when self pollinated, will give rise to kernels containing levels of oil in the range of 2.5 to 5.1 percent on a dry weight basis. This level of oil is typical of a wide range of field corn inbreds and hybrids. The term "maize" represents any variety, cultivar, or population of *Zea mays* L. "Male sterile" refers to plants which fail to produce functional pollen as a consequence of mechanical or hand detasseling, incorporation of genetic sterility, or by other mechanisms.

As used herein "nonisogenic" is a state of genetic dissimilarity between individual inbreds, hybrids or varieties.

As used herein, "normal corn" describes corn grain in which the oleic acid content of the oil ranges from 20–30% of the total fatty acids and the oil content is 2.5 to 5.1 percent on a dry weight basis.

As used herein in describing "oleic acid content", the term "high oleic" refers to a grain or seed having an oleic acid content of not less than about 50% of the total oil content of the seed, by weight measured at 0% moisture.

The "ovule" portion of the plant is a structure consisting of female reproductive tissue surrounded by maternal tissue. During the development of a corn plant the ovule will eventually house a haploid egg nucleus and two haploid polar nuclei. Following fusion with sperm nuclei found in pollen, the ovule will develop into a mature corn kernel. The "percent (%) oil" is the oil concentration of a corn kernel expressed on a dry weight basis. A "plant introduction" represents a collection of seeds or plants of the same species and region of origin which have been transported from one region to another. A large plant introduction collection is maintained by the United States Department of Agriculture. Plant introductions can be used by breeders as a source of genetic variation, but are not elite and often require extensive breeding to move desirable genes from the plant introduction into adapted varieties. The "pollen" is a structure which ultimately contains the two haploid sperm nuclei which fuse with the egg nucleus and polar nuclei found in the ovule to give rise to the embryo and endosperm of the mature corn kernel. A "population" is a genetically heterogeneous collection of plants sharing a common genetic derivation. The "seed" is the mature corn kernel produced for the purpose of propagating the species and for sale to commercial growers. A "synthetic" or "synthetic popula-

tion" is a genetically heterogeneous collection of plants of known ancestry created by the intermating of any combination of inbreds, hybrids, varieties, populations, races, or other synthetics. The terms "variety" or "cultivar" refer to a group of similar plants that by structural features and performance can be identified from other varieties within the same species.

Kernels from the plants of the present invention express a greater amount of oil and an improved oil composition relative to commercial varieties. The improvements relate to oxidative stability of the oil and to human health when the oil is used in food products, including its use as a cooking oil. The fatty acid profile of oil extracted from these varieties is dramatically different from the profiles seen in currently grown elite corn varieties.

Applicants teach a method for producing the novel corn varieties of the present invention and teach a method for producing high yielding elite varieties having substantial increases in oleic acid content.

EXAMPLES

The present invention is further defined in the following Examples. It will be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. The present invention can be used for any purpose where its properties are useful such as in, but not limited to, foods, frying oils, animal feeds, pharmaceuticals, and industrial oils. In the below Examples, where oil percents are expressed as percents of seed weight, zero percent moisture is presumed. Oleic acid content in Tables 1–5 is expressed as a percent of the total oil content of the seed. The calculation of oleic acid content as a percent of seed weight is carried out by multiplying the percent oil by the percent oleic acid.

Example 1

Breeding Methodology for Production of High Oil, High Oleic Germplasm

This example illustrates the creation of maize germplasm having a ten fold increase in oleic acid content over normal maize. Maize plants capable of producing oil having nearly three times the oleic acid content of normal maize were produced using a pollen mutagenesis technique described by M. G. Neuffer and E. H. Coe Jr., Maydica, 1978, 23:21–28. Two genotypes were mutagenized, and high oleic mutants were recovered in both. Maize plants of the background B73, an inbred line developed at Iowa State University and available to the public, and AEC272, a high oil line developed by the University of Illinois and licensed for commercial use exclusively by E. I. du Pont de Nemours and Company and Pfister Hybrid Corn Company were mutagenized. Pollen was collected from field-grown plants and sifted to remove anthers. Pollen was suspended and stirred constantly for 30 minutes in a solution of paraffin oil and ethyl methane sulfonate (EMS). Three concentrations of EMS were used for B73OL, 0.053%, 0.0625%, and 0.083%. One concentration, 0.0625%, was used for AEC272OL. The treated pollen was then brushed onto silks using a small paint brush.

Seeds (kernels) that developed on ears pollinated with treated pollen were subsequently germinated and plants were self pollinated. The seeds resulting from these plants were planted an additional time and self pollinated. Kernels were collected from individual self pollinated ears of B73 and analyzed using a Tecator™ Infratech Model 1255 near infrared transmission (NIT) spectrophotometer calibrated to detect kernels with elevated oleic content (Tecator AB, Box 70, S-263 21, Hoganas Sweden; Williams, P. C., 1987, Commercial Near Infrared Reflectance Instrumentation, In: Near Infrared Technology in the Agricultural and Food Industries; Williams, P. C. and C. Norris, eds. American Association of Cereal Chemists). A sample of kernels from those ears that were identified as bearing high oleic kernels by NIT were subjected to gas chromatography to further quantify their oleic acid content. Kernels from one ear averaging two to three times the oleic acid content of normal corn following gas chromatography were given the inbred line designation B73OL. The inbred family ACE27-2OL was similarly identified by analysis of kernels from original self pollinated ears of ACE272, although the NIT screen was omitted.

To accurately determine the oleic acid content, oil was extracted from 30 mg of ground corn using the following protocol:

1. Finely ground corn was placed in a 13×100 mm screw top tube.
2. 0.25 ml of a working solution of sodium methoxide was added. The working solution contains 20 ml of a 25% sodium methoxide added to 200 ml of methanol.
3. 1.0 ml of hexane was added.
4. The sample was mixed on a rotary shaker for 30 minutes.
5. 0.1 ml of 10% acetic acid was added.
6. The sample was vortexed, then centrifuged for 5 minutes at 2500 RPM.
7. Hexane was removed and the sample was placed in a gas chromatograph vial.
8. Fatty acid composition of the samples was determined using a Hewlett-Packard Model 5890 gas chromatograph.

At least 20 bulked kernels per F3 ear were used to determine oil and fatty acid composition. Oil produced by certain plants developed using this mutagenesis protocol contains approximately 60% oleic acid, or two to three times oleic acid produced in the non-mutant versions of these two genotypes.

Genetics studies with the high oleic B73 mutant showed that elevated oleic content in this line is conferred by a single gene which displays additive inheritance. Plants carrying the mutant gene were cross pollinated with plants of the background ASKC28, a high oil variety developed by the University of Illinois and licensed for commercial use exclusively by E. I. du Pont de Nemours and Company and Pfister Hybrid Corn Company. ASKC28 is a population of corn plants ranging in oil content from 7–22% and averages 18% oil. Average oleic acid content as a percentage of the oil in ASKC28 ears is 43%, and no ears in this population have been found to be above 50% oleic acid. F1 plants of this cross were self pollinated and F2 seeds were planted and the resulting plants were self pollinated to give a population of 956 F3 ears. Seed oil content was determined for F3 ears using a Tecator™ Infratech Model 1255 near infrared spectrophotometer calibrated against standard gravimetric oil determinations (Tecator AB, Box 70 S-263 21, Hoganas Sweden; Williams, P. C., 1987, Commercial Near Infrared Reflectance Instrumentation, In: Near Infrared Technology in the Agricultural and Food Industries; Williams, P. C. and C. Norris, eds. American Association of Cereal Chemists). For calibration purposes, the total oil concentration of shelled kernels was determined gravimetrically according to Method 920.39 of the Association of Official Analytical Chemists. Oleic acid content was determined by gas chromatography.

Of the 956 F3 ears, 49 had oil contents in the 15–20% range. Of these 49 ears, 3 had oleic contents above 60%, and 3 had oleic contents from 55–60% (Table 1). These oleic levels are higher than any that have been seen in the ASKC28 population, and are in the range of those seen in the B73 EMS derived mutant. Ear number 1125.04 had an oil content of 17.3% and an oleic acid content of 59.5%, which represents approximately a 10 fold increase in the total oleic acid as a percentage of the seed compared to normal corn.

Plants were grown from selection 1125.04 and were cross pollinated with another F3 selection of the same pedigree having an oil content of 15% and and oleic acid content of 59%. Plants arising from this cross were self pollinated and ears were analyzed for oil and oleic acid content. By crossing these two high oil, high oleic lines together, it was possible to create kernels having about 20% oil and about 60% oleic acid (Table 2).

These findings show that the mutant gene that confers the high oleic trait in the low oil corn line B73 is also effective in reducing the conversion of oleic acid to linoleic acid in seeds that produce over 5 times more oil. These results were unexpected assuming that the mutant gene confers a partial block in the oil biosynthetic pathway from oleic to linoleic acid. Until the discovery of the present invention, it was not known whether the total oleic acid as a percent of the seed would be greater than the total oleic acid percent of the B73 mutant which is low in oil. Organisms can find ways to circumvent blocks in biochemical pathways by using other enzymatic pathways. It was not known that the distribution of fatty acids, with particular reference to oleic acid, could be essentially the same for a seed that produces 17 to 20% oil as it is for a seed that produces only 4% oil.

TABLE 1

Oil and Oleic Acid Content of the 49 Highest Oil F3 Ears from a Population of 956 F2 Plants of the Cross ASKC28 × B73OL

	EAR ID NO.	% OLEIC	% OIL
1	1008.04	64.1	15.4
2	1048.01	60.7	15.0
3	1022.05	60.5	15.6
4	1125.04	59.5	17.3
5	1056.05	57.5	15.0
6	1015.06	56.1	15.7
7	1131.11	51.0	15.0
8	1041.01	47.6	17.4
9	1009.04	47.4	16.7
10	1058.03	47.2	15.7
11	1117.07	46.9	16.2
12	1084.06	46.5	20.0
13	1024.01	46.4	17.7
14	1089.04	46.0	16.2
15	1108.04	45.1	17.5
16	1096.02	44.3	15.7
17	1031.03	43.7	17.3
18	1031.01	43.3	20.0
19	1047.11	43.2	17.6
20	1063.07	42.8	15.4
21	1047.04	42.6	16.2
22	1118.07	42.6	15.8
23	1030.07	42.5	16.1
24	1100.03	42.2	18.4
25	1059.02	41.9	16.0
26	1072.02	41.5	15.5
27	1028.07	39.7	17.2
28	1028.07	39.7	16.6
29	1066.01	39.6	16.6
30	1096.05	39.4	17.3
31	1103.06	39.4	16.0
32	1014.05	38.4	16.0
33	1045.09	38.1	15.4
34	1006.10	38.1	15.1
35	1098.07	37.9	20.3
36	1118.04	37.6	17.4
37	1043.06	37.4	17.2
38	1020.01	37.0	18.4
39	1048.06	37.0	16.1
40	1101.01	36.7	20.3
41	1066.09	36.6	16.2
42	1101.03	36.4	19.2
43	1101.02	35.7	20.7
44	1101.04	35.2	22.4
45	1090.01	34.5	15.0
46	1109.01	33.6	15.3
47	1083.03	32.8	15.2
48	1111.02	30.4	17.1
49	1030.04	29.0	15.4

TABLE 2

Oil and Oleic Acid Content of S1 Ears Derived from a Cross Between Two S3 ASKC28 × B73OL Lines Having 15–17% Oil and 60% Oleic Acid

	EAR ID NO.	% OIL	% OLEIC
1347	BAR06360	14.99	58.6
1347	BAR06361	14.48	61.0
1347	BAR06362	13.80	60.9
1347	BAR06363	18.08	60.6
1347	BAR06364	12.25	58.8
1348	BAR06376	17.36	60.5
1348	BAR06377	17.57	58.2
1348	BAR06378	10.57	63.7
1348	BAR06379	10.64	59.5
1348	BAR06380	13.16	60.9
1348	BAR06381	15.96	58.9
1348	BAR06382	11.65	63.2
1348	BAR06383	15.90	60.4
1348	BAR06384	16.81	59.2
1348	BAR06385	13.95	62.1
1348	BAR06386	11.59	61.0
1348	BAR06387	16.37	58.8
1348	BAR06388	10.65	56.3
1348	BAR06389	13.60	54.8
1348	BAR06390	16.53	56.2
1349	BAR06401	16.76	60.6
1349	BAR06402	17.28	57.2
1349	BAR06403	12.58	52.4
1349	BAR06404	16.55	62.2
1349	BAR06405	17.07	60.7
1349	BAR06406	15.53	55.1
1349	BAR06407	15.19	58.9
1349	BAR06408	15.61	63.3
1349	BAR06409	14.99	58.0
1349	BAR06410	19.49	61.1
1349	BAR06411	17.19	61.6
1349	BAR06412	17.99	60.4
1349	BAR06413	13.80	53.4
1349	BAR06414	18.39	62.3
1349	BAR06415	17.03	61.2
1350	BAR06427	19.09	61.4
1350	BAR06428	19.14	64.1
1350	BAR06429	17.01	57.5
1350	BAR06430	17.62	64.5
1350	BAR06431	17.53	63.7
1350	BAR06432	20.21	58.1
1350	BAR06434	17.72	59.2
1350	BAR06435	18.02	60.9
1350	BAR06436	19.29	62.7
1350	BAR06437	16.90	61.2

TABLE 2-continued

Oil and Oleic Acid Content of S1 Ears Derived from a Cross Between Two S3 ASKC28 × B73OL Lines Having 15–17% Oil and 60% Oleic Acid

	EAR ID NO.	% OIL	% OLEIC
1350	BAR06438	18.54	60.0
1350	BAR06439	19.75	61.9
1350	BAR06440	14.38	62.9
1351	BAR06451	14.75	53.8
1351	BAR06452	13.00	59.4
1351	BAR06453	12.43	57.4
1351	BAR06454	14.97	58.1
1351	BAR06455	17.47	58.4
1351	BAR06456	11.27	62.5
1351	BAR06457	12.17	56.2
1351	BAR06458	12.45	60.5
1351	BAR06459	13.84	59.1
1351	BAR06460	16.58	56.6
1351	BAR06461	18.38	60.0
1351	BAR06462	15.28	60.6
1352	BAR06476	11.43	61.5

Example 2

Production of High Oil, High Oleic Acid Corn Grain

This example illustrates the production of agronomically elite, high yielding corn plants which bear grain having approximately a five fold increase in oleic acid content over normal corn. Maize plants of the genotype B73OL were pollinated by plants of the genotype AEC272OL to produce F1 seed. F1 hybrid plants of this cross were either self pollinated or cross pollinated by F3 plants obtained from samples 1008.04, 1022.05, 1048.01, and 1125.04 listed in Table 1. Self pollinated ears derived from samples 1008.04, 1022.05, 1048.01, and 1125.04 varied for oil and oleic acid content from ear to ear, which is not unexpected for F3 generation plants. One F3 derived ear was 17.1% oil and 67% oleic acid, indicating that the present discovery is repeatable under very different environmental conditions. The initial discovery of samples 1008.04, 1022.05, 1048.01, and 1125.04 was made in plants grown during the summer in Newark, Del. The following generation which gave rise to plants with 15 to 17% oil and 60 to 65% oleic acid was grown in the winter in Molokai, Hi. Self pollinated F1 plants of the cross B73OL X AEC272OL produce grain having an oil content of approximately 6% and an oleic acid content of the oil of approximately 60%. When cross pollinated by F3 plants derived from samples 1008.04, 1022.05, 1048.01, and 1125.04, grain produced on female F1 B73OL X AEC272OL plants averages 8.4% oil and 62.8% oleic acid. Oil and oleic acid levels of individual ears comprising this grain are shown in Table 3. Oil and oleic acid content are similar for grain produce in this manner under different environmental conditions. Data presented in Table 3 represent grain produced in the winter in Molokai, Hi., and bulked grain produced in the same manner in the summer near Newark, Del. averaged 9.6% oil and 64.1% oleic acid.

TABLE 3

Oil and Oleic Acid Content of Grain Resulting from the Cross Pollination of B730L × AEC2720L Ears by Pollen From F3 Derived Plants of Samples 1008.04, 1022.05, 1048.01, and 1125.04

	EAR ID NO.	POLLEN SOURCE[1]	% OLEIC	% OIL
1	1001.001 × 1000	1125.04	63.3	8.4
2	1001.002 × 1000	1125.04	58.8	7.6
3	1001.003 × 1000	1125.04	61.5	7.5
4	1001.004 × 1000	1125.04	65.8	8.4
5	1001.005 × 1000	1125.04	62.0	6.8
6	1002.001 × 1000	1125.04	65.3	9.2
7	1002.002 × 1000	1125.04	60.8	7.2
8	1002.003 × 1000	1125.04	63.4	7.5
9	1002.004 × 1000	1125.04	59.8	7.3
10	1002.005 × 1000	1125.04	60.9	8.5
11	1003.001 × 1000	1125.04	64.8	8.7
12	1003.002 × 1000	1125.04	61.4	7.1
13	1003.003 × 1000	1125.04	57.8	7.7
14	1003.004 × 1000	1125.04	63.0	8.2
15	1003.005 × 1000	1125.04	64.6	8.1
16	1004.001 × 1000	1125.04	62.6	7.7
17	1004.002 × 1000	1125.04	51.5	7.6
18	1004.003 × 1000	1125.04	63.8	8.2
19	1004.004 × 1000	1125.04	63.4	8.2
20	1004.005 × 1000	1125.04	66.5	8.3
21	1005.001 × 1000	1125.04	61.4	7.9
22	1005.002 × 1000	1125.04	65.7	8.6
23	1005.003 × 1000	1125.04	65.6	10.1
24	1005.004 × 1000	1125.04	64.0	11.0
25	1005.005 × 1000	1125.04	65.1	9.2
26	1007.001 × 1000	1125.04	61.1	7.4
27	1007.003 × 1000	1125.04	61.3	7.8
28	1007.004 × 1000	1125.04	60.7	8.4
29	1007.005 × 1000	1125.04	53.4	7.4
30	1008.001 × 1000	1125.04	52.2	7.1
31	1008.002 × 1000	1125.04	54.7	8.6
32	1008.003 × 1000	1125.04	62.2	9.1
33	1008.004 × 1000	1125.04	51.3	8.4
34	1008.005 × 1000	1125.04	61.7	9.1
35	1009.001 × 1000	1125.04	62.3	7.6
36	1009.002 × 1000	1125.04	63.0	7.5
37	1009.003 × 1000	1125.04	65.3	7.0
38	1009.004 × 1000	1125.04	58.6	9.6
39	1009.005 × 1000	1125.04	61.6	8.1
40	1010.001 × 1000	1125.04	61.3	9.9
41	1010.002 × 1000	1125.04	64.3	8.5
42	1010.003 × 1000	1125.04	61.4	8.0
43	1010.004 × 1000	1125.04	62.5	8.3
44	1010.005 × 1000	1125.04	48.0	8.5
45	1011.001 × 1000	1125.04	47.0	10.2
46	1011.002 × 1000	1125.04	60.2	7.8
47	1011.003 × 1000	1125.04	59.4	7.7
48	1011.004 × 1000	1125.04	52.3	8.0
49	1011.005 × 1000	1125.04	60.3	8.3
50	1013.001 × 1012	1022.05	66.2	7.2
51	1013.002 × 1012	1022.05	64.0	9.2
52	1013.005 × 1006	1008.04	63.1	7.4
53	1013.006 × 1006	1008.04	60.3	7.6
54	1013.007 × 1006	1008.04	64.5	6.9
55	1014.003 × 1012	1022.05	62.4	7.6
56	1014.004 × 1012	1022.05	62.7	7.7
57	1014.005 × 1012	1022.05	61.4	8.0
58	1015.002 × 1012	1022.05	62.3	7.6
59	1015.003 × 1012	1022.05	63.7	6.8
60	1015.004 × 1012	1022.05	62.9	7.0
61	1016.002 × 1012	1022.05	54.5	7.5
62	1017.001 × 1000	1125.04	46.7	8.8
63	1017.002 × 1000	1125.04	60.6	8.9
64	1017.003 × 1000	1125.04	58.1	8.1
65	1017.004 × 1000	1125.04	60.0	7.8
66	1017.008 × 1018	1048.01	62.4	7.6
67	1019.001 × 1018	1048.01	64.4	6.1
68	1019.002 × 1018	1048.01	63.2	7.4
69	1019.003 × 1018	1048.01	62.7	6.8
71	1019.005 × 1018	1048.01	64.9	7.9
72	1020.002 × 1018	1048.01	63.2	7.9

TABLE 3-continued

Oil and Oleic Acid Content of Grain Resulting from the Cross Pollination of B73OL × AEC2720L Ears by Pollen From F3 Derived Plants of Samples 1008.04, 1022.05, 1048.01, and 1125.04

	EAR ID NO.	POLLEN SOURCE[1]	% OLEIC	% OIL
73	1020.003 × 1018	1048.01	59.0	6.7
74	1020.004 × 1018	1048.01	61.5	6.4
75	1020.005 × 1018	1048.01	64.8	7.9
76	1020.006 × 1018	1048.01	65.1	7.1
77	1021.002 × 1018	1048.01	59.2	6.7
78	1021.003 × 1018	1048.01	66.8	7.5
79	1021.004 × 1018	1048.01	62.5	7.5
80	1021.006 × 1018	1048.01	63.6	8.0
81	1021.007 × 1018	1048.01	53.2	7.9
82	1022.001 × 1018	1048.01	64.5	7.7
83	1022.002 × 1018	1048.01	62.4	7.2
85	1022.004 × 1018	1048.01	62.8	6.5
86	1022.005 × 1018	1048.01	61.6	8.0
87	1023.002 × 1018	1048.01	62.8	7.6
88	1023.003 × 1018	1048.01	65.3	8.1
89	1023.004 × 1018	1048.01	62.8	7.1
90	1023.005 × 1018	1048.01	64.6	7.3
91	1023.007 × 1018	1048.01	62.2	8.3
	AVERAGE		62.8	8.4

[1]Pollen source identifies the F3 plants derived from F3 ears described in Table 1.

In this example, the grain parent (B73OL X AEC2720L), when self pollinated, will produce grain with an oil content approximately two percentage points higher than normal corn (6% vs. 4%). The oil level obtained in grain described in Table 3 is due in part to the increased oil contributed by the grain parent. By using pollinator plants of the type described in this example, high oil, high oleic grain can also be produced on grain parents that, if self pollinated, would produce normal oil levels (i.e., 4%). To illustrate this point, plants of the background B73OL were pollinated by plants of the background LH60 (a product of the Holden's Foundation Seed Co., Williamsburg, Iowa) to produce F1 hybrid seed. LH60 is an inbred line having a normal (4%) oil level and higher than normal oleic acid level (35–40%). Self pollinated grain produced on B73OL X LH60 plants is 3–4% oil and 45–50% oleic acid. Yield tests have shown that the hybrid B73OL X LH60 is capable of matching or exceeding the grain yield of current elite hybrids. Grain having an oil content of 8.7% and an oleic acid content of 56.8% was produced by allowing B73OL X LH60 F1 plants to be pollinated by a bulk of F4 high oil, high oleic plants from the cross B73OL X ASKC28 described in Example 1. The bulk of F4 pollinator plants represented 165 F4 ears derived from 20 F3 ears that had an average oil content of 15.0% oil and 57% oleic acid. Grain parent and pollinator plants were planted in a repeating pattern of six grain parent rows to two pollinator rows. In maize, the male part of the plant is the tassel which can be easily removed by hand or machine.

Female grain parent plants were detassled by hand and the resulting grain arose from wind-born pollen from pollinator rows. Approximately 2000 pounds of grain was produced in this manner. However, to facilitate cross pollination on a commercial scale, the plants to be used as the female would be rendered male sterile. This can be accomplished by physical removal of the male pollen-shedding part of the plant, by chemical treatment, or by a genetic mechanism such as cytoplasmic male sterility. Grain yields comparable to those of fully male-fertile hybrid plants can be achieved by planting a mixture of seeds containing a small percentage of pollinator and a large percentage of male sterile grain parent, for example wherein the ratio of the pollinator to the male sterile female grain parent is approximately one to six.

This example illustrates the important point that the present invention offers a way to produce a high yield of grain with substantial increases in oleic acid content over normal corn. Also, this grain production method dramatically reduces the breeding timeline by allowing growers to utilize currently available high-yielding corn hybrids in combination with selected pollinators to produce grain with substantially higher oleic acid content.

Example 3

Use of High Oil, High Oleic Corn in an Animal Feed Ration as a Means of Improving Meat Quality

By replacing some or all of the supplemental animal fat in a feed ration with the oil present in high oil, high oleic corn, it will be possible to produce meat products having less saturated fats. As discussed in the Background, the utility of this concept has been demonstrated in feeding trials with swine that were fed diets containing high oleic oil. In this example, a method of producing animals having less saturated fat and more monounsaturated fat by using a the present invention will be described. An important difference between this and the feeding trials described in the Background is that in this example, high oleic acid oil is supplied in the grain rather than as a supplement to the feed, offering greater convenience to animal producers. Feeding trials which have shown that adding oleic acid to the feed improves carcass quality typically use high oleic canola or sunflower oil. From a commercial standpoint, providing oleic acid in the grain rather than as a supplemental oil eliminates the cost, inconvenience, and time of handling an additional feed ingredient.

A protocol has been developed to evaluate the effects of feeding a high oil, high oleic corn type on several factors including:

A. the growth of swine,

B. the fatty acid composition of fat and muscle tissue,

C. the stability of carcass fat,

D. consumer preference of improved meat products.

Four feeding programs would be utilized to raise swine with improved carcass composition. Feeding Program 1 consists of a corn and soybean meal diet which contains no added fat. Feeding Program 2 consists of a high oil, high oleic corn plus soybean meal diet in which the relationships between critical nutrients on the caloric density are equal to those of Feeding Program 1. Feeding Program 3 consists of a corn and soybean meal diet with added crude corn oil formulated to achieve the same caloric density and nutrient to calorie relationships as Feeding Program 2. Feeding Program 4 consists of a corn and soybean meal diet with added animal fat formulated to achieve the same caloric density and nutrient to calorie relationships as Feeding Program 2.

To evaluate any interaction between hog genetic background and the above feeding programs, two hog types would be used in this study. Group 1 consists of hogs having a high productive performance and high lean gain potential with a mature body weight of over 240 pounds. Group 2 consists of hogs with a "classical" genetic potential for lean gain and productive performance with a mature body weight of under 220 pounds. The total number of experimental treatments for this phase of the experiment is 8, and includes 4 feeding programs and 2 hog types.

Diets need to be formulated for each treatment feeding program for each of four production stages. The production stages, based upon live body weight are as follows:

a. 45–90 pounds

b. 90–130 pounds

c. 130–200 pounds

d. 200–240 pounds

Four pens per treatment, and six pigs per pen, for a total of 192 pigs, are needed. At the beginning of the trial, pigs are to be weighed and allotted to pens, within a genetic potential treatment, in a manner which equalizes weight and sex across feeding program treatment. Pigs within each pen need to similar in weight and proportion of females versus barrows.

Hogs need to be weighed at the beginning of each production stage and at the end of the feeding period. The weight of feed added to the feeders in each pen must be recorded as it is added. Feed remaining in the feeder at the end of each production stage must be removed and weighed. Hogs are to housed in a confinement facility and feed and water are to be offered ad-libitum. Hogs within a genetic background treatment are to be sent to slaughter when they reach their mature body weight.

Hogs are to be slaughtered and processed in a commercial plant which purchases hogs on the basis of grade and yield. Tissue samples from the ham and loin, and the subcutaneous fat surrounding these cuts must be obtained and saved for analysis. Samples of ham and loin from 1 pig from each pen is to be processed and saved for sensory evaluation.

Feeding performance can be evaluated by comparing the average daily gain, the average daily feed intake, and the feed efficiency (pounds of feed/pounds of gain) for each of the treatments. The effect of the treatments on carcass quality can be evaluated by measuring average carcass weight, average back fat, average percent lean yield, and average actual yield. The effect of the treatments on meat quality can be evaluated by analyzing the ham, loin, and the surrounding subcutaneous fat for fatty acid profile, oxidative stability, and meat firmness. Consumer related factors such as taste and appearance of ham and loin cuts must be evaluated by a trained and experienced sensory panel.

Example 4

Improved Functional Properties of Oil Extracted from High Oil, High Oleic Corn

This example illustrates the improved oxidative stability and subsequent utility of oil extracted from high oil, high oleic corn. Crude oil was extracted from the grain samples described in Example 2, and from a bulk of normal corn hybrids. For each sample, approximately 4 pounds of clean grain was cracked using a Rosskamp model TRC-650-6 cracking roller. Oil extraction was done in a glass extraction vessel heated to 60° C. in a water jacket. Two gallons of hexane was added to the cracked grain in the extraction vessel, and the solvent cycled through the system for 45 minutes. After extraction, hexane was removed with a rotary evaporator, leaving crude oil. Corn oil used in commercial cooking applications or in food products is not used in its crude form, but rather is refined, bleached, and deodorized. The crude oil was processed using procedures designed to mimic those used by commercial manufactures of refined corn oil. Commercial conditions cannot be duplicated exactly on a laboratory scale. However, the conditions and procedures employed approximate those used commercially.

To refine, bleach, and deodorize the oil, 300 g of crude oil was placed in a 600 ml glass beaker and 0.3 g of H_3PO_4 was added dropwise while stirring as a 0.1% solution of 85% H_3PO_4. The sample was heated to 65–70° C. and held for 10 minutes. Warm (60° C.) NaOH (8%) was added dropwise to the oil sample to neutralize the free fatty acids and the H_3PO_4. The sample was stirred for 5 minutes, then split among centrifuge tubes and centrifuged for approximately 5 minutes at 2500 RPM. The soap film was swabbed from the top of the tube and oil was decanted into a clean beaker. The oil was then water washed with the addition of 20% (v/v) of hot water as the sample was heated to 90° C. with rapid agitation. The oil and water were allowed to stand and separate for 10 minutes and the sample was centrifuged again at 2500 RPM for 10 minutes. Oil was decanted into a small beaker to prevent the accidental contamination of the oil with the aqueous phase and then was poured into a 500 ml suction flask. The oil was dehydrated using very rapid agitation under vacuum at 85–95° C. for 30 minutes or until all moisture (bubbles, condensation) had been removed. The vacuum was then broken with nitrogen. Two percent (wt/wt) of Filtrol F-160 was added and the vacuum was again immediately applied slowly with rapid agitation for and additional 30 minutes at 85–95° C. While under vacuum, the oil was allowed to cool to 60° C. with reduced agitation. The vacuum was then broken with nitrogen and one percent (wt/wt) of diatomaceous earth was dispersed in the oil. The mixture was suction filtered through a prepared bed of diatomaceous earth supported by filter paper into a 500 ml suction flask.

Following refining and bleaching, the oil was deodorized in a deodorization vessel. The first trap of the vessel was filled with ice and water, the second trap was filled with liquid nitrogen. 4 ml of deionized water was added per 100 g of oil in the boiler portion of the vessel. Two drops of 25% citric acid were added to the vessel, then oil was added to the citric acid solution in the vessel. The sample was heated to 240° C. under vacuum and nitrogen flow at one PSI. Nitrogen flow was stopped when the sample reached 60° C. At completion of the deodorization process, the sample is cooled to 40° C. and the refined oil was removed to a flask.

Oleic acid percent of oil extracted from the samples was measured by gas chromatograph. The oleic acid level represents an average of four 20 g subsamples of grain.

Oil oxidation occurs in two stages, the first being the induction period, and the second being the exponential phase; Lin, S. S., Fats and Oils Oxidation, In: Introduction to Fats and Oils Technology, P. J. Wan, ed., American Oil Chemists Society, Champaign, Ill., pp. 211–231. Oxidation of an oil proceeds through free radical formation, hydroperoxide formation, and oxidation products formation. The first detectable products of oxidation are hydroperoxides. Once formed, the peroxides will begin to decompose and form volatile and non-volatile oxidation products. The volitile compounds generally have objectionable odors and can be smelled easily, the smell being one of rancidity or staleness. The non-volatile products can be further classified as polarized and polymerized compounds.

The length of time needed to produce a rapid acceleration of oxidation (the exponential phase) of oils and fats is indicative of resistance to oxidation. This length of time is measured as a mathematical determination of the maximum change of the rate of oxidation, and is known as the Oil Stability Index (OSI).

An OSI instrument, Omnion, Inc., Rockland, Mass., was used to predict the stability or resistance to oxidation by measuring the rate of volitile compound formation. Method Cd 12b-92 of the American Oil Chemists Society was used. Cleaning of the poly carbonate tubes and the conductivity probes accomplished with RBS 35 (FLUKA) and hot water.

5.0 g±0.2 g of oil sample was placed in disposable sample tubes. All connections were made according to the manufacturers directions and the samples were run at 110° C. Water supply is a Barnstead NANOpure II system with Type I, Organic Free and Pyrogen Free cartridges. Duplicates of each sample were performed in the same run. Placement of tubes in the OSI instrument was accomplished in a random fashion. Data were collected and OSI was determined on a DELL computer using software supplied by the manufacturer. Oils with high induction times as measured by the OSI are more resistant to oxidation than oils with low values (Table 4).

Heat accelerates oxidation of oils and alters the pattern of oxidation products. The temperature of an oil is generally kept between 180° and 250° C. during deep fat frying. At such temperatures, the oil will oxidize at a faster rate and the nature of oxidation will be different from the the nature of oxidation at room temperature. At frying temperatures, the formation of polymerized molecular species is generally greater. As a result, the oil will develop a dark color, have higher viscosity, and will foam easily. The polar and polymer indecies, as used here, reflect an oils ability to withstand heat and remain stable. A high index reflects rapid breakdown of the oil and rapid accumulation of polymeric and polarized breakdown products. The lower the index, the greater the ability of the oil to withstand the elevated temperatures used in frying applications.

The system for the analysis of the high temperature stability tests consists of a heating unit, a Rainin Instrument Dynamax HPLC and data acquisition system, two Rabbit-HP solvent delivery pumps, ICI Insurument's AS 2000 auto injector, and MiltonRoy spectromonitor UV detector at 254 nm. Five ml of oil per sample was placed in a 13×100 mm glass screw cap test tube. The tubes were placed in an aluminum heating block that holds 13 mm tubes. The aluminum block was heated by a Thermolyne type 1900 hot plate that is controlled with a PMC Dataplate 520 temperature controller with timer. This provides a consistent temperature of 180° C. in the aluminum block and provides auto off of the hot plate after ten hours. After 10, 20, 30, and 40 hours of heating time (10 hours per day over a 4 day period), a 50 microliter sample was removed and placed in a 2 ml screw cap HPLC vial with Teflon and silica septa for polar and polymer analysis.

Samples were placed in a −20° C. freezer until assayed. Just prior to HPLC analysis, samples were brought to room temperature. 950 microliters of hexane and 1.5% isopropyl alcohol were added and the samples were vortexed. Samples were placed in the autosampler of the HPLC for assay.

The HPLC column used is a Beckman Ultrasphere 4.6×25 cm. The method used is similar to that used by Lin; supra; who looked at the oxidation of soybean oil at elevated temperatures. The mobile phases were, Reservoir A: isopropanol, and Reservoir B: methanol. Starting conditions were 2 minutes at 40% A and 60% B, followed by a linear gradient change to 70% A and 30% B over a 7.5 minute time period. This was held for 4.5 minutes and then the gradient was changed linearly to 90% A and 10% B over 2.5 minutes. This was held for 2.5 minutes and then the gradient was changed linearly to 95% A and 5% B over 2.5 minutes and held for 22.5 minutes before returning to starting conditions over 5 minutes.

Resulting chromatographs were integrated and areas for polar and polymeric peaks in the samples were determined. The increase in polar and polymeric material over heating times was plotted and fitted with a regression equation. The resulting equation was integrated from 0 to 40 hours to determine the total area under the regression curves. The number representing the total area under the curve was given the name of polar index or polymer index. Table 5 shows polar and polymer indecies for normal corn oil and oil obtained from high oil, high oleic corn. Oils having lower polar or polymer indecies are more stable. The polarized and polymerized compounds that are generated during heating are the main causes of foaming and bitter taste during prolonged deep fat frying. Foods fried in oxidized oil become stale rapidly and have a short shelf life.

TABLE 4

Induction Time of Oil Obtained from Normal Corn and High Oil, High Oleic Corn Described in Examples 1 and 2

Sample	% Oleic Acid	Induction Time (Hrs.)	Induction Time Standard Deviation
Normal Corn[1]	30	6.1	0.07
ASKC28OL[2]	58	10.7	0.32
(B73OL × AEC272OL) × ASKC28OL (Newark, DE, 1993)	64	11.5	1.34
(B73OL × AEC272OL) × ASKC28OL (Molokai, HI, 1993)	63	13.2	0.28
(B73OL × LH60) × ASKC28OL	57	10.8	0.28
LSD (0.05)		1.6	

[1]Normal corn consists of blended grain from four commercially grown hybrids.
[2]ASKC28OL represents a bulk of F4 high oil, high oleic plants from the cross B73OL × ASKC28 described in Example 1. The bulk of F4 pollinator plants represented 165 F4 ears derived from 20 F3 ears that had an average oil content of 15.0% oil and 57% oleic acid.

TABLE 5

Polar and polymer indecies of oil obtained from normal corn and high oil, high oleic corn described in Examples 1 and 2

Sample	% Oleic Acid	Polar Index ± Std. Dev.	Polymer Index ± Std. Dev.
Normal Corn[1]	30	187 ± 14	1078 ± 35
ASKC28OL[2]	58	142 ± 8	532 ± 71
(B73OL × AEC272OL) × ASKC28OL (Newark, DE, 1993)	64	121 ± 12	470 ± 25
(B73OL × AEC272OL) × ASKC28OL (Molokai, HI, 1993)	63	143 ± 21	528 ± 45
(B73OL × LH60) × ASKC28OL	57	182 ± 23	543 ± 33
LSD (0.05)		43	115

[1]Normal corn consists of blended grain from four commercially grown hybrids
[2]ASKC28OL represents a bulk of F4 high oil, high oleic plants from the cross B73OL × ASKC28 described in Example 1. The bulk of F4 pollinator plants represented 165 F4 ears derived from 20 F3 ears that had an averagel oil content of 15.0% oil and 57% oleic acid

Example 5

AEC272OL and B73OL can be Used to Produce Inbred Lines of Corn Expressing Elevated Levels of Oleic Acid

Tables 6 and 7 demonstrate that B73OL and AEC272OL can be used effectively in a breeding program to increase the

oleic acid content of many different corn inbreds. The data in Table 6 are taken from the most recently analyzed cycle of a partially completed backcross breeding program. Backcrossing is a conservative breeding method which is most often used to introduce simply inherited, highly heritable traits into existing agronomically elite inbred lines. In a typical backcrossing program involving a quality grain trait (i.e., a trait which influences the composition of a corn kernel, such as waxy), one or a series of varieties containing a quality grain trait are crossed to a series of elite inbred lines, which are termed recurrent parents. The progeny of these crosses are again crossed back to their respective recurrent parents, and this cycle is repeated typically 5 to 8 times. During this process the quality grain trait is maintained in each backcrossing project by visual or other selection, and the average nuclear genetic composition of each emerging inbred becomes closely similar to that of the elite recurrent parent. The result is the production of a series of elite inbred lines which express the newly introduced grain quality trait and in all other respects very closely resemble the plant type and combining characteristics of their respective recurrent parents. Finally, these finished inbreds are selfed and homozygous individuals selected so the quality grain trait is uniformly expressed in subsequent seed increases.

B73 as a line has given rise to a large number of elite female corn inbreds widely employed in commercial production today. Further, B73 is closely related to several of the inbreds employed as recurrent parents in Table A. This further increases the probability that elite inbreds will be recovered from backcrossing projects employing B73OL as a source of the high oleic trait. While not as elite as B73, AEC272 is a well adapted experimental high oil male inbred and performs reasonably well in prototype high oil hybrids, suggesting that inbreds derived from AEC272OL after backcrossing should again yield agronomically elite inbreds.

The data in Table 6 gives the range of oleic values observed in the selfed progeny of a number of backcross conversion projects in which AEC272OL or B73OL were used as donors of the high oleic oil trait. The level of oleic acid in these projects was determined by selfing partially backcrossed lines during each backcrossing cycle, bulking the resultant kernels (termed S1) individually by ear, and measuring the fatty acid composition of a representative sample of kernels by gas chromatography using a slight modification of the extraction and analytical methods described in Example 1. Due to the crossing protocol employed, and because oleic content in both AEC272OL and B73OL is most likely controlled by a single semidominant gene with additive effects, we expect the genes causing high oleic acid content to segregate in these populations and thus expect a broad range of oleic acid content across S1 ears in projects that continued to carry the high oleic traits. In contrast, if the high oleic acid trait was either not expressed in a particular genetic background or was lost by faulty selection during the backcrossing process, we would expect a typically narrow range of oleic content in the kernels from selfed ears, as is typical of inbred lines of corn. In this analytical run the range of oleic content seen upon analysis of several ears of each of the recurrent parent inbreds was typically about 5%.

TABLE 6

Percent Oleic Acid in Oil Extracted from Lines Undergoing Backcross Conversion Growth in 1994 in Molokai, Hawaii

Recurrent Parent	Oleic Source	Backcross Generation (n)	% Oleic in BC(n) S1 Range	Average	Observations
LH59	AEC272OL	4	44–20	32.4	20
LH60	AEC272OL	4	53–31	41.9	8
LH61	LH105 × B73OL	4	33–27	30.4	4
LH74	LH105 × B73OL	4	44–25	32.0	11
LH82	AEC272OL	4	42–21	26.8	14
LH85	AEC272OL	3	45–25	33.0	19
LH132	B73OL	3	57–23	37.7	12
LH132	LH105 × B73OL	4	40–23	28.3	18
LH145	LH105 × B73OL	4	54–22	31.8	16
LH146	B73OL	4	33–22	26.5	11
LH150	LH105 × B73OL	3	26	26.4	1
LH163	AEC272OL	4	43–29	34.0	12
LH168	LH82(4) × AEC272OL	0	57–24	42.4	11
LH169	LH82(4) × AEC272OL	0	53–24	36.9	13
LH172	LH82(5) × AEC272OL	0	48–20	31.5	15
LH172	LH105 × B73OL	3	39–23	33.1	6
LH185	LH59(5) × AEC272OL	0	43–22	31.1	8
LH186	LH59(5) × AEC272OL	0	33–20	25.7	10
LH192	LH105 × B73OL	4	58–37	43.8	16
LH192	AEC272	4	61–37	46.2	14
LH195	LH105 × B73OL	3	55–27	32.3	18
LH197	LH105 × B73OL	4	62–27	39.0	19
LH198	LH105 × B73OL	3	50–37	44.0	4
LH199	132(5) × B73OL	0	40–29	34.4	2
LH200	LH105 × B73OL	4	43–26	34.6	5
LH206	LH150(5) × LH150 × B73OL	0	43–24	31.4	10
LH206	LH105 × B73OL	4	47–30	38.1	9
LH211	LH105 × B73OL	4	35–21	26.1	8
LH212	LH216(5) × AEC272OL	0	31–20	25.1	10
LH213	LH216(5) × AEC272	0	45–25	34.4	13
LH213	LH18 × B73OL	3	58–33	46.1	8
LH216	AEC272OL	4	36–24	30.5	6

TABLE 6-continued

Percent Oleic Acid in Oil Extracted from Lines Undergoing Backcross Conversion Grown in 1994 in Molokai, Hawaii

Recurrent Parent	Oleic Source	Backcross Generation (n)	% Oleic in BC(n) S1 Range	Average	Observations
LH218	LH216(5) × AEC272OL	0	31–18	24.3	11
LH219	LH216(5) × AEC272OL	0	34–25	28.8	9
LH223	B73OL	4	45–28	34.2	10
LH225	LH18 × B73OL	4	51–26	34.3	15

Out of the 36 backcross projects presented in Table 6 17 exhibited a range of oleic acid contents of 20 percentage points or greater, while 16 exhibited a range of oleic acid content of 11 percentage points or greater. Of the remaining projects LH150 returned only one ear during this cycle and hence failed to show a range of segregation, and the oleic trait may have been lost during the course of the LH61 and LH219 backcross projects. Overall, these results indicate that B73OL and AEC272OL can be used as effective donors of the high oleic acid trait, and that the high oleic trait is expressed at sufficiently high levels in a number of genetic backgrounds to allow simple, effective selection during backcross breeding.

Oil extracted from B73OL and AEC272OL kernels typically exhibits oleic acid levels of 60%. These levels of oleic acid are generally not seen in Table 6 because none of the S1 ears examined are expected to be homozygous for the oleic genes present in either AEC272OL or B73OL. To gain an estimate of the final oleic acid level which may be achieved in finished lines after backcross conversion, plants from the second backcross generation (BC2) from several backcross projects were self pollinated to yield a BC2S1 kernels. Ears bearing BC2S1 kernels exhibiting elevated oleic contents when bulked were replanted in a field in Newark, Del. in the summer of 1994 and resultant plants were self pollinated to yield BC2S2 kernels. It was expected that a proportion of these BC1S2 plants would be homozygous for the high oleic gene present in B73OL and AEC272OL and that kernels obtained from BC2S2 ears from these homozygous plants would be uniformly high in oleic acid content. Kernels produced on the remainder of the BC2S2 ears would either exhibit oleic acid levels typical of corn inbreds or would contain a mixture of grain types. The upper range of oleic acid content seen in BC2S2 ears should thus be indicative of the expression of kernels uniformly expressing the high oleic trait. Since approximately 87% of the nuclear genome of BC2S2 kernels should be derived from the recurrent parent, these oleic levels should be generally representative of the oleic acid content of oil extracted from seeds of the finished inbreds when these backcrossing projects are completed.

TABLE 7

Percent Oleic Acid in Oil Extracted from Bulked BC2S2 Kernels Produced in Newark, Delaware During Summer 1994

Recurrent Parent	Oleic Source	Percent Oleic, BC2S2 Range	Average	Observations
LH59	AEC272OL	64–24	40.4	49
LR60	AEC272OL	59–43	43.6	39
LR61	LR105/B73OL	62–24	39.6	44

TABLE 7-continued

Percent Oleic Acid in Oil Extracted from Bulked BC2S2 Kernels Produced in Newark, Delaware During Summer 1994

Recurrent Parent	Oleic Source	Percent Oleic, BC2S2 Range	Average	Observations
LH74	LR105/B73OL	62–26	40	16
LH82	AEC272OL	58–23	38.1	55
LH85	AEC272OL	61–23	36.7	51
LR132	LH105/B73OL	63–23	40.22	51
LH132	B73OL	58–22	39	50
LR145	LH105/B73OL	67–22	37.3	43
LH146	B73OL	64–22	40.8	48
LH150	LH105/B73OL	53–27	39.1	15
LR163	AEC272OL	58–26	39.5	45
LR172	LH105/B73OL	60–24	36.4	34
LR192	LH105/B73OL	70–32	52	46
LR192	AEC272OL	70–25	53.2	31
LR195	LH105/B73OL	61–22	35.6	55
LR197	LH105/B73OL	60–24	40.7	56
LR198	LH105/B73OL	68–26	42.6	53
LH200	LH105/B73OL	62–25	39.9	56
LR206	LH105/B73OL	50–24	35.6	44
LH211	LR105/B73OL	55–24	33.8	31
LH212	LR105/B73OL	62–22	41.8	34
LH213	LR16 × B73OL	56–28	39.6	25
LH216	AEC272OL	47–25	32.2	33
LH223	B73OL	48–26	36.2	28
LH225	LR18 × B73OL	48–30	41.3	20

Out of the 26 backcross projects examined in Table 6 19 returned ears with kernels containing oil with an oleic acid content of 57% or greater. Four of these projects contained oil with an oleic acid content of 65% or greater, and the two backcross projects involving LH192 as recurrent parent returned ears whose kernel oil contained 70% oleic acid. Table 7 further illustrates that it is possible to recover high oleic segregants in the LH61 project and hence the failure to recover high oleic LH61 segregants in Table 6 is more likely due to missed selection during the latter stages of the backcrossing program rather than by any suppressive effect of the LH61 background.

Overall, it appears that both AEC272OL and B73OL will be effective in producing inbred lines of corn by the backcross method of breeding which in most cases will produce kernels whose oil will contain approximately 60% oleic acid. In some genetic backgrounds the final percentage of oleic acid is likely to approach 70%. It is further likely that breeding methods other than those employed during backcross conversion will also be effective in producing new inbreds containing oil which in many cases contains approximately 60% oleic acid, because the inheritance of the oleic trait from these sources is simple, the trait is highly selectable, and does not exhibit a high enough degree of either genotypic or environmental variability to adversely

effect selection. Since the oil of selfed kernels from the hybrid B73OL X AEC272OL is itself 60% oleic acid, it is very likely that inbreds derived from either B73OL or AEC272OL can by combined in hybrid combination to produce hybrid corn grain containing oil with approximately 60% oleic acid content.

Example 2 teaches the production of grain containing oil with an oleic acid content of approximately 60% when utilizing various combinations of AEC272OL, B73OL, ASKC28OL, and standard corn inbreds. The data presented in Example 5 suggest that similar combinations made between suitably selected inbreds derived from either AEC272OL or B73OL and the high oil, high oleic pollinator ASKC28OL or derivatives will similarly produced grain containing approximately 6.5 to 10% oil, which oil has a content of approximately 60% oleic acid.

What is claimed is:

1. A corn grain produced by planting in close proximity a corn plant of an agronomically elite high-yielding female parent, having high oleic characteristics, and optionally having high-oil characteristics, with a corn plant of a high-oil and high oleic male parent, optionally having high-yielding characteristics and/or agronomically elite characteristics wherein said corn grain has an oil content of at least 6.5% on a dry weight basis and further wherein said oil is comprised of not less than about 55% oleic acid and the total oleic acid content of the grain is about 3.6% to about 7% of the total seed weight.

2. The grain of claim 1 wherein the high-oil, high oleic male parent plant, when self or sib pollinated, is capable of producing kernels having a total oil content ranging from 7.5% to 20% of the total seed weight, measured at zero percent moisture and an oleic acid content of not less than about 55% of the total oil content of the seed.

3. The grain of claim 1 wherein the agronomically elite female parent, when self or sib pollinated, is capable of producing kernels having a total oil content of between about 2 percent to about 7.5 percent of the total seed weight, measured at zero percent moisture, wherein the oleic acid content is not less than about 55% of the total oil content.

4. The corn grain of claim 1 wherein the high-oil, high oleic male parent, when self or sib pollinated, is capable of producing kernels having a total oil content ranging from 7.5% to 20% of the total seed weight, measured at zero percent moisture and an oleic acid content of not less than about 55% of the total oil content of the seed and wherein the agronomically elite female parent, when self or sib pollinated, is capable of producing kernels having a total oil content of between about 2 percent to about 7.5 percent of the total seed weight, measured at zero percent moisture, wherein the oleic acid content is not less than about 55% of the total oil content.

5. The grain of claim 4 wherein the female parent has an oil content of of not less than 6% of the total seed weight, measured at zero percent moisture.

6. The grain of claim 4 wherein the oleic acid content is about 3% to about 7% of the total seed weight.

7. Progeny plants and plant parts from any pedigree derived from the corn grain of claim 1.

8. Progeny plants and plant parts from any pedigree derived from the corn grain of claim 2.

9. Progeny plants and plant parts from any pedigree derived from the corn grain of claim 4.

10. Corn plants and the seed thereof regenerated from a tissue culture of the plant or plant parts selected from the group consisting of claims 7, 8, and 9.

11. The grain of claim 1, claim 3 or claim 4 wherein the female parent that is crossed to produce such grain is rendered male sterile by chemical, mechanical, or genetic means.

12. The corn grain of claim 4 wherein the high oleic characteristics of both the female plant and the male plant are generated from a high oleic corn inbred line designated B73OL which bears the ATCC accession number 97026.

13. The corn grain of claim 4 wherein the high oleic characteristics of both the female plant and the male plant are generated from a high oleic corn inbred line designated AEC272OL which bears the ATCC accession number 97027.

14. The corn grain of claim 4 wherein the high oleic characteristics of the female plant are generated from a high oleic corn inbred line designated B73OL which bears the ATCC accession number 97026 and the high oleic characteristics of the male plant are generated from a high oleic corn inbred line designated AEC272OL which bears the ATCC accession number 97027.

15. The corn grain of claim 4 wherein the high oleic characteristics of the female plant are generated from a high oleic corn inbred line designated AEC272OL which bears the ATCC accession number 97027 and the high oleic characteristics of the male plant are generated from a high oleic corn inbred line designated B73OL which bears the ATCC accession number 97026.

16. The corn grain of claim 1, wherein the corn grain borne by the female parent plant only, produced from the planting in close proximity, is selectively harvested, where such corn grain is substantially free of grain produced by self pollination of the female.

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Lipid Bodies

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1 Introduction

Most seeds contain storage lipids in the form of triacylglycerols, which usually comprise 20–50% of the total seed dry weight (Appelquist 1975; Gurr 1980; Roughan and Slack 1982). This lipid reserve is rapidly mobilized to provide energy and carbon skeleton for the growth of the embryo during germination. The triacylglycerols are densely packed in subcellular organelles called lipid bodies (oleosomes, spherosomes, oil bodies). The spherical lipid body is about 1 μm in diameter, and is surrounded by a half-unit membrane of about 3 nm thickness (Fig. 1; Yatsu and Jacks 1972). The fatty acid moieties of the membrane phospholipids are believed to orient themselves toward the matrix so that they can form hydrophobic interaction with the internal triacylglycerols.

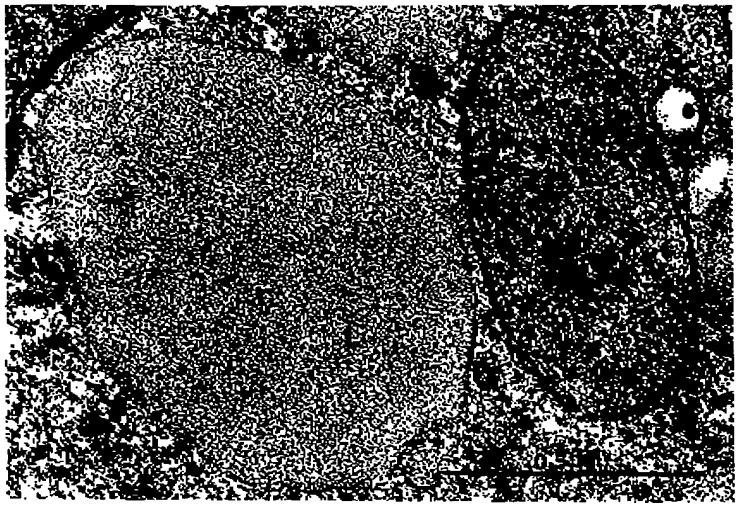

Fig. 1. Lipid body (*L*), microbody (*MB*), and small portion of a plastid (*P*), representing organelles surrounded by "half-unit" membrane, single membrane (one "unit" membrane, tripartite), and double membrane, respectively, in the subapical zone of a 1-day-old shoot apex of corn seedling. (Courtesy of R. N. Trelease 1969)

2 Ontogeny

The ontogeny of the lipid bodies is still unclear. In maturing seed, the lipid bodies do not contain any enzyme for triacylglycerol biosynthesis. Instead, the fatty acids are synthesized in the plastids (Appelquist 1975; Gurr 1980; Roughan and Slack 1982). The subsequent formation of mono-, di-, and tri-acylglycerols from activated fatty acids occurs in the endoplasmic reticulum. The mechanism of transport of fatty acids from the plastids to the endoplasmic reticulum, and of triacylglycerol from the endoplasmic reticulum to the lipid bodies is unknown. The origin of the lipid body membrane is also unclear. It has been suggested that the newly synthesized triacylglycerols in the endoplasmic reticulum are sequestered between the two phospholipid layers of the membrane at a particular region so that the hydrophobic triacylglycerols are stabilized by hydrophobic interactions (Schwarzenbach 1971, Wanner and Theimer 1978). Continuous deposition of the newly synthesized triacylglycerols at the same region eventually generates a budding vesicle of triacylglycerol surrounded by a half-unit membrane. The vesicle is then detached to become a lipid body. An alternative postulation states that the lipid bodies arise directly in the cytoplasm by condensation of triacylglycerol following by formation of the surrounding membrane (Bergfeld et al. 1978).

In seed germination, the triacylglycerols are hydrolyzed to fatty acids and glycerol in the initial step of mobilization (Galliard 1980; Huang 1984). In many seeds, lipase activity appears during germination concomitant with the decrease in the triacylglycerols. The lipase is associated with the membrane of the lipid bodies. The fate of the membrane components after the depletion of triacylglycerols is still unknown.

3 Isolation

Lipid bodies have an equilibrium density of less than 1.09 $g \cdot cm^{-3}$, and therefore can be isolated by flotation centrifugation of the tissue homogenate. When isolation is performed with mature seeds in which the lipid bodies contain no detectable lipase activity (see Sect. 4), the procedure can be performed at room temperature. When germinating or mature seeds are used, the procedure should be performed on ice or in a cold room (4 °C). The endosperm of castor bean, or the storage tissues of various other oil seeds in mature or germinating stage can be used.

The grinding medium contains 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, and 0.15 M Tricine buffer adjusted to pH 7.5 with KOH (Moreau et al. 1980). Dehulled castor bean is chopped with a new razor blade in grinding medium (15 g in 30 ml) on a Petri dish, and ground gently with a mortar and pestle. The homogenate is filtered through a piece of Nitex cloth (Petko, Elmsford, N.Y., USA) of pore size 20 μm × 20 μm or eight layers of cheesecloth. Each 15 ml of the filtrate is placed in one 40-ml centrifuge tube, and 15 ml flotation medium (grinding medium containing 0.5 M instead of 0.6 M sucrose) is layered on top. After centrifugation at 10,000 *g* for 10 min, the lipid bodies float to the top and are removed with a spatula. The lipid pad is carefully resuspended in 15 ml grinding medium with a rubber policeman by gentle stirring and pressing the unresuspended lumps against the tube wall. The 15-ml resuspended lipid bodies is again placed in a centrifuge tube, and a similar flotation centrifugation is performed. The procedure is repeated one more time (a total of three times flotation centrifugation). The final lipid pad is resuspended in 3-ml grinding medium.

4 Markers of Lipid Bodies

Naturally, triacylglycerols are the most important marker of the lipid bodies. There are no unusual phospholipid components in the membrane of the lipid bodies. The major phospholipids are phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol (Moreau et al. 1980). On the contrary, the lipid body membrane contains unique protein components as revealed in SDS polyacrylamide gel electrophoresis (Bergfeld et al. 1978; Moreau and Huang 1979; Roughan and Slack 1982). If necessary, these protein components can be used as markers of the membrane, although the assay is tedious and nonquantitative.

Lipase activities may be present in the membrane of lipid bodies isolated from germinating seeds. The activity has been found in castor bean, corn, mustard, rape, and cotton (Huang 1984; Lin and Huang 1983; Moreau et al. 1980; Ory 1969; Ory et al. 1960). In other seeds such as soybean, sunflower, peanut, and cucumber (Huang 1984), the lipase activity is not detectable, and its absence may be due to the occurrence of inhibitors or components interfering with the enzyme assay. In those seeds with lipid bodies having detectable lipase activities, the enzyme may be tightly (castor bean, corn) or loosely (rape, mustard, and cotton) associated with the membrane. In the latter case, most of the enzyme in association with a small fraction of the membrane can be readily washed away from the isolated lipid bodies by simple grinding media. The removable lipase-containing membrane fraction has been postulated to represent either an extension of the lipid body membrane on which nascent lipase is synthesized (Wanner and Theimer 1978), or remnants of those activated lipid bodies in which the triacylglycerols have been hydrolyzed (Bergfeld et al. 1978).

Two exceptions are known in which the lipid bodies isolated from mature (desiccated) seeds contain lipolytic enzyme activities. In castor bean, the lipase is synthesized together with the lipid bodies in seed maturation, and persists throughout desiccation and germination until the triacylglycerols have been depleted (Moreau et al. 1980; Muto and Beevers 1974; Ory 1969; Ory et al. 1960). In soybean, an active acyl hydrolase which acts on monoacylglycerols but not di- and tri-acylglycerols is present in the mature seed (Lin and Huang 1982). During germination, its activity rapidly disappears before the depletion of the triacylglycerols. The function of the soybean hydrolase is unknown.

Several other enzymes are present in the lipid bodies of seeds. However, they are not unique to the lipid bodies or they have very restricted occurrence. NADH-

Cytochrome c oxidase is present in trace amounts in the lipid body membranes (Moreau et al. 1980). This enzyme does not seem to serve any metabolic role; its presence probably reflects the origin of the lipid body membrane directly or indirectly from the endoplasmic reticulum. Jojoba seed is unique among oil seeds because it is the only oil seed known to contain intracellular wax esters instead of triacylglycerols as food reserve. In the membrane of the lipid bodies (wax bodies) from jojoba seedlings, a wax esterase, a fatty alcohol oxidase, and a NADH-fatty aldehyde dehydrogenase are present (Moreau and Huang 1979). These enzymes catalyze the hydrolysis and conversion of storage wax esters to fatty acids.

The lipid body membrane can be obtained from the isolated lipid bodies (Jack et al. 1967; Moreau et al. 1980; Ory et al. 1960). Diethyl ether apparently can penetrate the membrane and solubilize the matrix triacylglycerol. To 6 ml of the resuspended lipid bodies (from 30 g castor bean) in a tube equipped with a Teflon screw cap, 6 ml diethyl ether is added. After shaking, the ether is removed by aspiration. The extraction is repeated two more times. After the third extraction, a stream of nitrogen is passed into the preparation in order to evaporate the remaining ether. After the extraction, about 50% of the membrane remains intact and the rest is solubilized (Moreau et al. 1980). The whole preparation can be used for further study. Alternatively, the preparation can be centrifuged at 100,000 g for 2 h to separate the membrane in the pellet and the solubilized components in the supernatant. If desired, the membrane fraction can be viewed under the electronmicroscope (Fig. 2; Jack et al. 1967; Trelease 1969).

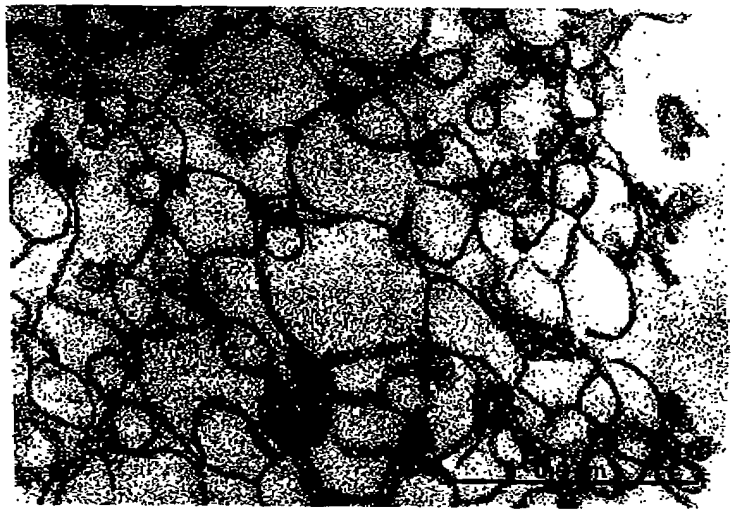

Fig. 2. Isolated lipid body membrane of corn scutella. The membranes appear as tripartite structures which are interpreted to represent closely appressed half-unit membranes of lipid bodies after the matrix triacylglycerols had been extracted. (Courtesy of R. N. Trelease 1969)

5 Assays

Two methods of assaying the marker enzyme lipase are described. The first method is a fluorometric assay of acyl hydrolase activity using an artificial substrate (Lin and Huang 1983; Muto and Beevers 1974), and the other method is a colorimetric assay of true lipase activity (Lin and Huang 1983; Nixon and Chen 1979). A true lipase (EC 3.1.1.3) is a glycerol ester hydrolase that releases fatty acids from insoluble triacylglycerols. The fluorometric method is a very convenient assay for the activities of lipase, as well as nonlipase acyl hydrolase, which is present in other compartments of the tissue. Thus, the assay is not specific, and should only be used on lipid bodies after they have been isolated (e.g., in subfractionation of the protein components). The colorimetric method is relatively time-consuming but measures only the true lipase activity.

5.1 Fluorometric Assay

Acyl hydrolase activity is measured at room temperature in a reaction mixture of 4 ml, containing 0.1 M Tris-HCl buffer, pH 7.5 (depending on specific enzyme), 2 mM dithiothreitol, and enzyme preparation. The reaction is initiated by the addition of 0.1 ml of 3.3 mM (final concentration, 0.83 mM) N-methylindoxylmyristate (from I.C.N. Pharmaceuticals, Cleveland, Ohio, USA) dissolved in ethylene glycol monomethyl ether. Fluorescence measurements are made with a Turner Model 111 fluorometer with excitation filter No. 405 (405 nm maxima) and emission filter No. 2A–12 (> 510 nm) attached to a recorder. The reaction is usually linear for the first 10 min. Although unnecessary, a more sophisticated fluorometer can be used. The activity is expressed on a relative basis of ΔF unit per unit time.

In the past few years, the commercial supply of N-methylindoxylmyristate was unreliable. If it cannot be obtained, fatty esters of 4-methylumbelliferone are used (Hasson and Lalies 1976). Many fatty derivatives of 4-methylumbelliferone are available commercially (from Sigma Corps., St. Louis, Mo, USA, and several other biochemical companies). Lipase from a particular seed species may exhibit some degree of specificity towards the fatty derivatives. Nevertheless, 4-methylumbelliferyl laurate is probably an active substrate of lipases from most if not all sources. In the assay, 1 mM of 4-methylumbelliferone laurate is used instead of 0.83 mM N-methylindoxylmyristate, and excitation filter No. 7–60 (365 nm Maxima) is used instead of No. 405. If desirable, the actual activity in nmol min^{-1} can be calculated from a standard curve of fluorescence units versus 4-methylumbelliferone (stable and commercially available) concentrations in the assay system minus enzyme at the same pH.

5.2 Colorimetric Assay

The true lipase from an individual seed species exhibits high activities on the native substrates which are present in the same seed species (Huang 1984). Most

seeds contain triacylglycerols of oleic or linoleic acids, and the lipase from these seeds are generally active on triolein or trilinolein. Therefore, either of the two substrates is used for the lipase assay. The substrate should be free of contaminating monoacylglycerols which are also substrates for non-lipase acyl hydrolases in the same tissues. The substrate preparation obtained commercially should be checked by thin layer chromatography for purity irrespective of the claims of purity made by the manufacturers. The substrate is dissolved in chloroform (5–10 μg/10 μl for each spot) and spotted onto a TLC plate coated with 250 μm of Silica Gel G (Brinkman Instruments, Inc., Westbury, N.Y., USA). The plate is developed in 50:50:1 (v/v/v) hexane:diethyl ether:acetic acid, and allowed to react with iodine vapor (put some crystals of iodine in a TLC tank). Standards of tri-, di-, and mono-olein/linolein and the free acid (5–10 μg/10 μl for each spot) are run on the same TLC plate. The mobilities of the components in descending order are triacylglycerol, fatty acid, 1,3-diacylglycerol, 1,2-diacylglycerol, and monoacylglycerols. A small percent of free fatty acid in the triacylglycerol preparation will not interfere with the assay. If monoacylglycerol is present, the triacylglycerol preparation is purified on a similar TLC plate. The triacylglycerol preparation is applied as a line across the whole plate (200 μg per one 20 cm × 20 cm plate). A similar but smaller plate (20 cm × 5 cm) is run in the same tank as a marker. After development, the small plate is allowed to react with iodine vapor. By making a comparison between the two plates, the position of triacylglycerol in the large plate can be identified. The silica gel containing the triacylglycerol line is scrapped and extracted with chloroform. After centrifugation to remove the silica gel, the chloroform supernatant is obtained, and the chloroform is evaporated.

The activity of true lipase is measured by a colorimetric method. Although the released fatty acids can be measured more rapidly using an automatic titrator, the colorimetric method has the advantage of requiring no specific equipment or setup. Furthermore, if many enzyme samples are to be assayed, the colorimetric method probably consumes about the same amount of time per assay, and all the assays can be performed simultaneously for a more uniform quantitation. In the colorimetric assay, the fatty acids produced are converted to copper soaps and measured using 2,2'-diphenylcarbazide. The reaction is performed at room temperature in a 5-ml tube. The 1-ml reaction mixture contains 0.1 M Tris-HCl, pH 7.5 (depending on specific enzyme), 5 mM dithiothreitol, 5 mM substrate, and enzyme preparation. Triolein or trilinolein (50 mM) is first emulsified in 2 ml of 5% gum acacia for 1 min at low speed with a Bronwill Biosonic IV ultrasonic generator fitted with a microprobe. The reaction is stopped at time intervals (ranging from 5 min, to 2 h, depending on the amount of enzyme used) to ensure that proper kinetics are observed. Each 0.1 ml aliquot of the reaction mixture is put in a 7-ml tube and boiled in a boiling water bath for 5 min. After cooling to room temperature, 4 ml of chloroform heptane methanol (4:3:2, v/v/v) is added. The tube is closed with a Teflon screw cap, and shaken horizontally for 15 min. Two ml of 0.1 M sodium phosphate, pH 2.5 is added, and the tube is shaken horizontally for 3 min. After centrifugation in a table-top centrigufe, the upper layer of methanol water is pipetted out and discarded. One ml of 0.01 M HCl is added, and the tube is shaken horizontally for 3 min. After centrifugation, the upper layer of HCl solution is pipetted out and discarded. One and half ml of copper reagent (0.1 M $Cu(NO_3)_2$, 0.2 M triethanolamine, 0.06 N NaOH, and 6 M NaCl) is added. The tube is closed with a Teflon screw cap, and shaken horizontally for 30 min. After centrifugation in a table-top centrifuge, 2 ml of the chloroform layer is transferred to a tube, and 0.1 ml of color reagent (10 ml freshly prepared 0.4%, 2,2'-diphenylcarbazide solution in 100% ethanol, plus 0.1 ml 1 M triethanolamine added immediately before use) is added. After 5 min or more, the absorbance is read at 550 nm. Oleic acid or linoleic acid is used to produce a standard curve that is linear up to a concentration of 0.05 μmol per 2 ml chloroform.

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Expression of Lipid Body Protein Gene during Maize Seed Development

SPATIAL, TEMPORAL, AND HORMONAL REGULATION*

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The storage lipids of seeds are packaged into lipid bodies, simple organelles containing a triacylglycerol core surrounded by a layer of phospholipids and proteins. A cDNA encoding the major protein from lipid bodies of maize (L3) was used as a hybridization probe to analyze the regulation of lipid body biosynthesis during seed development and germination. Immunofluorescent microscopy demonstrates that L3 protein accumulation is tissue-specific, confined to the embryo (scutellum and embryonic axis) and the aleurone layer of developing seed. Northern analyses show that L3 mRNA also accumulates to high levels in the embryo and is not found in any nonseed tissue. The steady-state level of L3 mRNA is developmentally regulated, increasing during early seed development and peaking at about the midpoint of seed development. It then decreases slowly, to 20% of the peak level, in the embryo of the mature seed and declines rapidly to undetectable levels as the seed germinates. The high level of L3 gene expression during seed development is not due to amplification of the L3 gene, since Southern analyses of maize genomic DNA indicate that the L3 gene has the same relatively low copy number throughout development (1–4 copies/haploid genome). Rather, the increase in the steady-state level of L3 mRNA during seed development is transcriptionally regulated. The developmental changes in L3 mRNA levels are paralleled by changes in the transcription of the L3 gene, as measured by run-off transcription in isolated nuclei. The role of the plant growth regulator abscisic acid in regulating L3 gene expression was investigated. When the mature seed is allowed to imbibe in the presence of exogenous abscisic acid, germination is retarded, and the period of L3 gene expression is extended. The extended period of L3 gene expression in the presence of exogenous abscisic acid is due, at least in part, to a dramatic and specific increase in L3 gene transcription.

Developing seeds accumulate large quantities of starch, protein, and lipid reserves which are stored in the dormant seed. These seed storage reserves are mobilized during germination to support the growth of the embryonic plant into an autotrophic seedling. The seed storage proteins from many plant species have been studied extensively. They are encoded by multigene families, and their expression is highly regulated with regard to tissue specificity and developmental timing (Refs. 1 and 2; for review, see Ref. 3). The regulation of α-amylase, which is involved in mobilization of the seed starch reserves, has also been intensively studied and is known to be under hormonal control at the level of gene transcription (4–6). In contrast to seed storage protein and starch metabolism, seed lipid metabolism has not been studied at the gene level, and the genetic mechanisms which regulate lipid deposition and mobilization during early development are unknown.

We have been using the maize seed as a model system to study the regulation of lipid metabolism during seed development and germination (7–10). In maize seeds, lipid reserves are synthesized and stored primarily in a specialized tissue of the embryo, the scutellum, where they constitute 50% of the dry weight at seed maturity. As in all seeds, the storage lipid in maize seeds is packaged into simple organelles called lipid bodies. These small spherical organelles consist of a triacylglycerol core surrounded by a "half-unit" membrane comprised of a single layer of phospholipids and a few major proteins (10–13). Only four major polypeptides are associated with lipid bodies of maize; and the smallest of these, called L3, is quite abundant and constitutes nearly 30% of the total lipid body protein and about 1% of the total protein of scutella. We recently cloned and sequenced an L3 cDNA and deduced the amino acid sequence of the L3 protein (14). Analyses of the L3 amino acid sequence suggest that this protein interacts with the single layer of phospholipids at the lipid body surface. However, the mechanisms which regulate the synthesis of L3 and other proteins involved in lipid body biosynthesis are not known.

In this study, we have addressed three questions to help elucidate the mechanisms which regulate L3 gene expression during maize development. Are L3 gene expression and protein accumulation confined to specific tissues? Is L3 gene expression differentially regulated during development; and if so, at what level(s) is it regulated? Is L3 gene expression affected by the plant growth regulator abscisic acid; and if so, at what level(s) is it affected? Our results indicate that L3 gene expression is tissue-specific and under both developmental and hormonal controls at the level of gene transcription.

MATERIALS AND METHODS

Plant Tissues—Seeds of the inbred line of maize (*Zea mays* L., Mo. 17) were obtained from the Illinois Foundation Seed Corp. (Champaign, IL). Tissues from developing seeds were obtained from field-grown plants. Plants were hand-pollinated, and seeds were collected at various time points during seed development. In germination and seedling studies, mature seeds were rinsed well in H_2O and incubated for 24 h at 20 °C while shaking at 120 rpm in a dish of water or abscisic acid solution ((±)-*cis,trans*-abscisic acid, Sigma) which barely covered the seeds. The seeds were transferred to Petri dishes contain-

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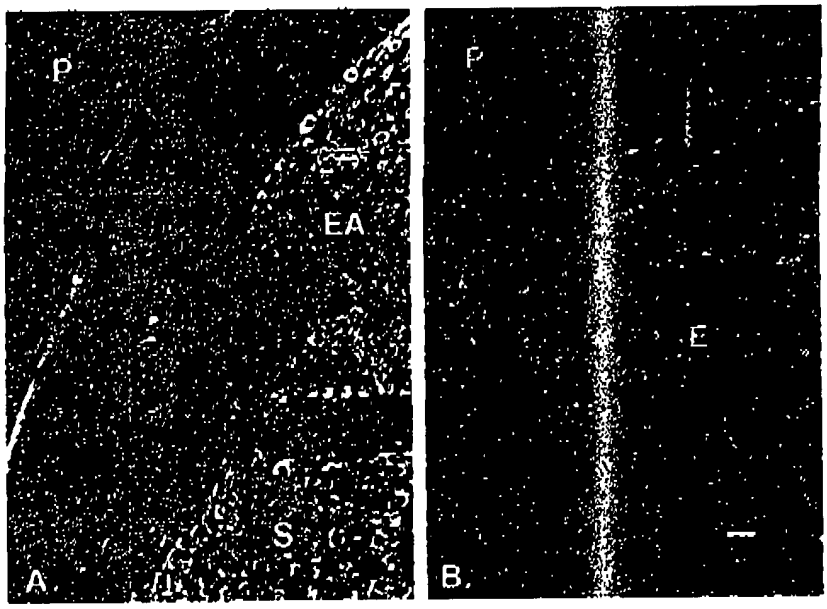

FIG. 1. **Immunofluorescent localization of L3 protein in maize seed.** *A*, longitudinal section through the embryonic axis and periderm of maize seed at ~30 DAP. L3 protein is evident in the cells of the embryonic axis and scutellum. *B*, longitudinal section through a portion of the periderm and endosperm. L3 protein is localized in the aleurone (indicated by the *arrow*), a single layer of cells at the periphery of the endosperm. *P*, periderm; *EA*, embryonic axis; *E*, endosperm; *S*, scutellum. The *bar* represents 10 μm.

ing Whatman No. 3MM paper saturated with either water or abscisic acid solution and were allowed to grow in darkness at 20 °C. Water or abscisic acid solution was changed daily. Unless stated otherwise, only the scutella, with the embryonic axes removed, of the seedlings were used. Radicle emergence from the seed coat was used as the criterium to determine seed germination.

Immunofluorescence—Free-hand longitudinal sections of maize seed at approximately 30 days after pollination (DAP)[1] were fixed in 100% ethanol/glacial acetic acid (95:5) overnight at −20 °C, dehydrated in a graded alcohol series, cleared in toluene, and embedded in paraffin. Sections 6–7 μm in thickness were deparaffinized and hydrated to phosphate-buffered saline (PBS) (66.7 mM Na_2HPO_4, 66.7 mM KH_2PO_4, 0.8% NaCl, pH 7.4). After preincubation for 30 min at 37 °C in PBS containing 5% preimmune goat serum, the sections were incubated at 37 °C for 60 min with rabbit serum containing L3 antibodies (diluted by 1:50 or 1:200) (7) or rabbit preimmune antiserum (1:50) as described (15). The sections were rinsed in PBS and incubated in a 1:33 dilution of fluorescein-tagged goat anti-rabbit IgG for 30 min at 37 °C. After rinsing with PBS, sections were viewed with a Reichert fluorescence microscope using transmitted UV light from a mercury arc source.

Northern Analyses—Total cellular RNA was isolated from various maize tissues, glyoxylated, fractionated by agarose gel electrophoresis, and blotted to Hybond N paper (Amersham Corp) (16). The blot was exposed to UV light for 3.5 min, processed, prehybridized, and hybridized as previously described (17). The 780-base pair L3 cDNA (14) was subcloned from λgt11 into the *Eco*RI site of plasmid pBR325. The pBR325-L3 plasmid was cut with *Eco*RI and the L3 cDNA insert was isolated from an acrylamide gel by high salt extraction, ethanol-precipitated, and nick-translated as previously described (17). The concentration of probe was 5 ng/ml in all hybridizations.

Southern Analyses—Genomic DNA was isolated from 3-day-old seedlings or embryos at 30 DAP by cetyltrimethylammonium bromide extraction and CsCl equilibrium ultracentrifugation exactly as described (18). The DNA was cut to completion with various restriction enzymes, fractionated by agarose gel electrophoresis (5 μg of DNA/lane), and blotted to Hybond N paper (Amersham Corp.) (17). The blot was processed and hybridized as described for Northern analyses. 0.5, 5, and 25 copy reconstructions of the L3 cDNA clone were included on each blot as a positive control for determination of the copy number of the gene in the maize genome. Gene copy reconstructions were based on a maize genome of 4×10^9 base pairs and on the assumption that maize has a diploid genome (19).

[1] The abbreviations used are: DAP, days after pollination; PBS, phosphate-buffered saline.

Transcription in Isolated Nuclei and Filter Hybridization—Embryos were isolated and homogenized in liquid nitrogen and then in buffer containing 0.44 M sucrose, 2.5% (w/v) Ficoll (*M*, 400,000), 5% (w/v) dextran 40 (*M*, 40,000), 25 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5% (w/v) Triton X-100, and 2 mM spermine. Nuclei were isolated exactly as described (20) by filtration through cheesecloth and nylon mesh, followed by Percoll gradient centrifugation. Nuclei were washed twice in buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 20% glycerol. DNA was measured by a fluorometric assay as described (21), except that the concentration of Hoechst 33248 reagent was reduced to 100 ng/ml. Nuclei were resuspended in transcription reaction buffer containing 16% glycerol, 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 150 mM NH_4Cl, 0.4 mM CTP, 0.4 mM GTP, 0.4 mM ATP, 1 mCi/μl [α-^{32}P]UTP (600 Ci/mmol), and 300 mM heparin (22). Heparin prevents reinitiation of transcription and also prevents RNA breakdown by inhibiting nuclease activity. After incubation at 33 °C for 8 min, 15 μg of carrier tRNA was added, and RNA was isolated as previously described (23). Filters for hybridization were prepared by applying 1 μg of various cloned DNAs or nonrecombinant plasmid DNA to Hybond N paper using a slot-blot apparatus. The maize alcohol dehydrogenase cDNA clone was the kind gift of Dr. David Ho (Washington University, St. Louis, MO). Filters were hybridized to the isolated ^{32}P-labeled transcripts and rinsed as previously described (24) and exposed to x-ray film. The autoradiograms were scanned with a Helena quick scan densitometer.

RESULTS

Tissue Specificity—The distribution of L3 protein in various tissues of the developing seed (30 DAP) was determined by immunofluorescent microscopy using L3-specific antibodies. Fig. 1*A* shows that the L3 protein was localized in both major parts of the embryo, the scutellum and embryonic axis. Furthermore, the protein was also detected in the aleurone layer, a nonembryo tissue that comprises the single outermost layer of endosperm cells (Fig. 1*B*). In contrast, little or no fluorescence was detected in other nonembryo tissues, the periderm and central endosperm (Fig. 1*B*). Similar seed sections incubated with preimmune rabbit antiserum showed low levels of nonspecific fluorescence (data not shown).

We further examined tissues of developing seeds (30 DAP) for the presence of L3 mRNA. The steady-state level of L3 mRNA was measured by RNA blot analysis using L3 cDNA

as a hybridization probe. Equal amounts of total RNA from the following three samples were analyzed: 1) isolated embryos (scutella and embryonic axes); 2) nonembryo seed tissues (embryos were removed from whole seeds, leaving periderm, endosperm, and aleurone tissues); and 3) nonembryo seed tissues (enriched in periderm and aleurone, with both the embryos and much of the central endosperm tissue removed from the whole seeds). The highest level of L3 mRNA was found in the isolated embryos (sample 1, Fig. 2, *lane 4*). However, L3 mRNA was also detected in nonembryo seed tissues (sample 2) at about 1% of the level in embryos (Fig. 2*B*, *lane 6*). When the sample was enriched in periderm and aleurone by removal of central endosperm (sample 3), the level of L3 mRNA increased to about 5% of that in the embryo (Fig. 2*B*, *lane 5*). These data indicate that L3 mRNA accumulates to a high level in embryos, but is also present in a subset of nonembryo seed tissues, presumably the aleurone. Thus, both L3 mRNA and L3 protein accumulate in the embryo of the developing seed.

We have also measured the steady-state levels of L3 mRNA in a variety of nonseed tissues to determine if the expression of the L3 gene is specific to seed tissues. L3 mRNA was not detected in any nonseed tissue examined, including pollen, silks, shoots and roots of 6-day-old seedlings, or unfertilized ovaries (Fig. 2*A*). Based on the sensitivity of the blot analysis, we estimate that the L3 mRNA level in each of these tissues is less than 0.5% of the highest level in seed tissue.

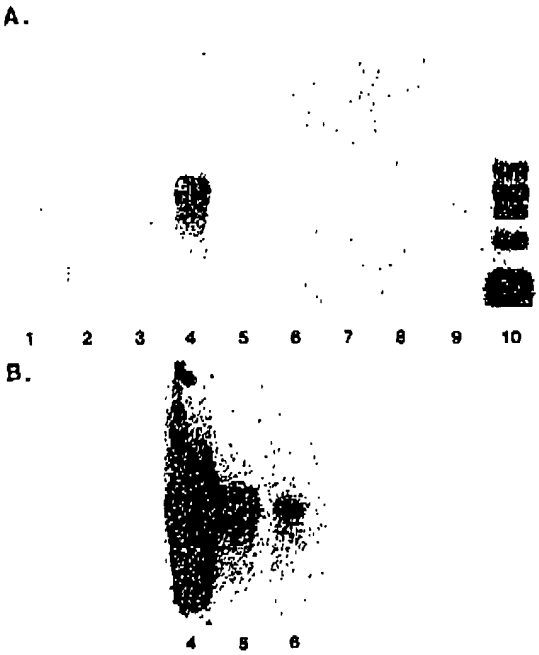

FIG. 2. **Tissue specificity of L3 mRNA.** A Northern blot of total RNA from the following maize tissues was hybridized to a nick-translated isolated insert of the L3 cDNA clone. *A*: *lane 1*, pollen; *lane 2*, growing silks; *lane 3*, unfertilized ovaries; *lane 4*, embryos at ~30 DAP (sample 1); *lane 5*, seeds minus embryos and most of central endosperm at ~30 DAP (sample 3); *lane 6*, seeds minus embryo at ~30 DAP (sample 2); *lane 7*, green shoots from 6-day-old seedlings; *lane 8*, roots from 6-day-old seedlings; *lane 9*, embryos from mature seed. *Lane 10* shows the migration of ^{32}P-labeled ϕX174 replicative form DNA cut with the restriction enzyme *Hae*III. The first four bands from *top* to *bottom*, respectively, are 1353, 1078, 872, and 603 base pairs long. *B*, a portion of the same blot shown above exposed to film 10 times longer to show minor L3 mRNA bands in *lanes 5* and *6*.

Temporal Regulation—The temporal pattern of L3 gene expression was examined by measuring the relative steady-state levels of L3 mRNA in maize embryos at different stages of seed development and germination. Total cellular RNA was examined by RNA blot analysis using equal amounts of total RNA per lane and L3 cDNA as a hybridization probe, and the results are shown in Fig. 3. The relative levels of L3 mRNA per unit of total RNA as determined by densitometric scanning of autoradiographs are plotted in Fig. 4*A*. The L3 mRNA levels were highest at approximately 25 DAP and declined slowly to approximately 20% of the peak level in mature seed embryos. L3 mRNA remained at approximately this same level during the first day post-imbibition and then declined precipitously to an undetectable level at day 2 post-imbibition. The message was not detected in the scutella at any later time point in seedling growth. These results indicate that the level of L3 mRNA accumulation is tightly regulated during seed development, germination, and early seedling growth.

No Amplification of the L3 Gene—L3 mRNA is an abundant message in the developing embryo, but is not present in detectable levels in the seedling and many maize tissues. It is possible that the high L3 mRNA level in the developing embryo is due to a developmentally timed and tissue-specific amplification of the L3 gene. This possibility was examined by comparing the L3 gene copy number in genomic DNA from developing embryos and seedlings. Genomic DNA was isolated from embryos at 30 DAP and 6-day-old seedlings. The DNA was cut to completion with the restriction enzyme *Eco*RI and analyzed by Southern blot analysis using L3 cDNA as probe. The L3 cDNA probe hybridized to the same four bands at approximately the same intensity in DNA from either developing embryos or seedlings (Fig. 5). The L3 gene copy number was estimated at 1–4 copies/haploid maize genome based on reconstruction with known amounts of cloned DNA (Fig. 5) and on similar analyses using five other restriction enzymes (data not shown). These analyses indicate that the L3 gene is present in the genome at a relatively low copy number and is not amplified or dramatically rearranged in developing embryos where the mRNA accumulates to a high level.

Transcription of L3 Gene during Seed Development—We examined the role of L3 gene transcription in regulating the developmental changes in steady-state levels of L3 mRNA. The relative level of L3 gene transcription at various stages of seed development was determined by run-off transcription in isolated nuclei. Embryos at four developmental stages (19, 26, 31, and 40–45 DAP) were isolated and frozen in liquid N_2. Nuclei were isolated by Percoll gradient centrifugation and incubated in the presence of [^{32}P]UTP under conditions which allow completion of nascent transcripts. Total incorporation of [^{32}P]UTP per unit of DNA in the transcription reaction as determined by trichloroacetic acid precipitation was fairly constant at the three earlier time points (85, 102, and 86 × 10^3 dpm/μg of DNA, respectively). However, total incorporation of [^{32}P]UTP per unit of DNA was reduced 3–4-fold (25 × 10^3 dpm/μg of DNA) in nuclei from older embryos (40–45 DAP). The decrease in total transcription in nuclei from older embryos probably reflects the fact that the seeds are dehydrating and becoming dormant at this developmental stage.

The relative level of L3 gene transcription in nuclei at each developmental stage was quantitated. Transcripts from each reaction were hybridized to L3 cDNA and nonrecombinant plasmid DNA bound to nylon paper. The relative level of hybridization to L3 cDNA was quantitated by autoradiography and densitometric scanning. Nonspecific hybridization to plasmid DNA was negligible. The relative level of L3 gene

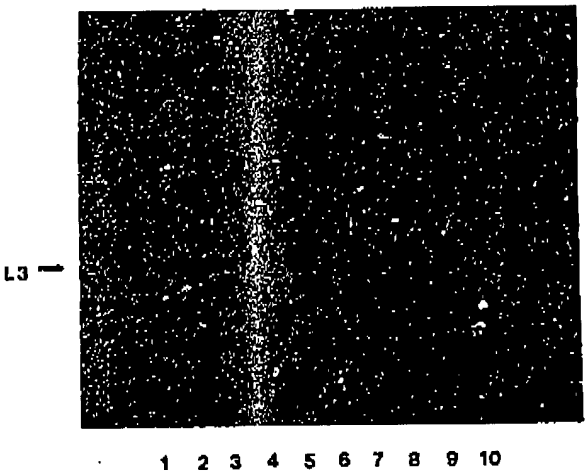

FIG. 3. **Temporal regulation of steady-state levels of L3 mRNA during maize seed development and germination.** A Northern blot is shown of total RNA (7 μg/lane) isolated from embryos at the following times during seed development and germination and hybridized to a nick-translated isolated insert from the L3 cDNA clone: *lane 1*, 18 DAP; *lane 2*, 25 DAP; *lane 3*, 31 DAP; *lane 4*, 40–45 DAP; *lanes 5–10*, 1 through 6 days, respectively, after imbibition.

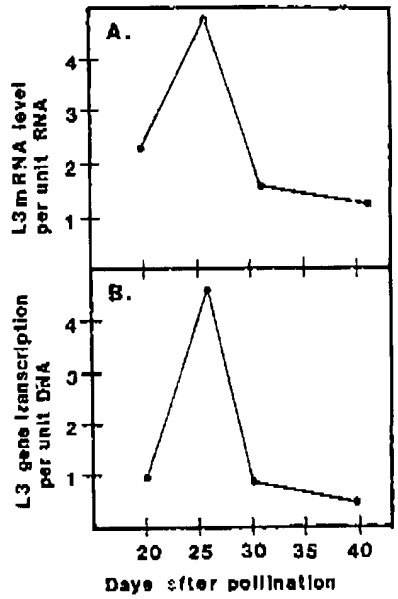

FIG. 4. **Time course of L3 mRNA accumulation and L3 gene transcription during seed development.** *A*, relative levels of L3 mRNA per unit of total RNA as determined by densitometric scanning of the Northern blot shown in Fig. 3; *B*, relative levels of L3 gene transcription per unit of DNA in run-off transcription assays using isolated nuclei from embryos at the four indicated times after pollination. Transcripts were hybridized to L3 cDNA bound to paper and quantitated by autoradiography and densitometric scanning. The amount of DNA in each transcription reaction was measured fluorometrically.

transcription per unit of DNA at each time point is plotted in Fig. 4*B*. At the three earlier time points, the relative level of L3 gene transcription changed nearly 5-fold, whereas the total transcription remained nearly constant. This suggests a

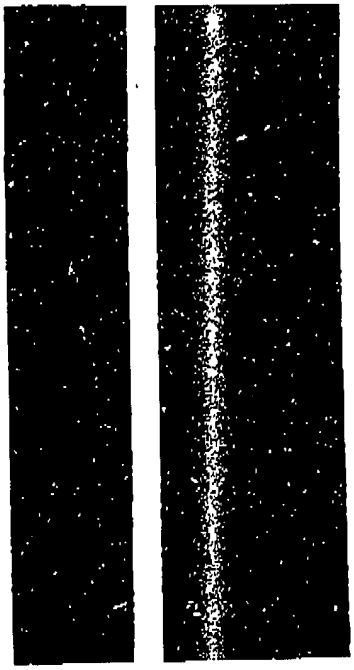

FIG. 5. **Southern analysis of L3 gene copy number in genomic DNA from embryos of developing seeds and young seedlings.** Southern blot analysis is shown of DNA isolated from developing embryos at ~30 DAP (*lane D*) or 6-day-old seedlings (*lane G*) and cut with the restriction enzyme *Eco*RI. Reconstructions showing 0.5, 5, and 25 gene copy equivalents/haploid maize genome were made with linearized L3 cDNA plasmid DNA and are shown to the *right*.

specific modulation of L3 gene transcription. At the latest time point (40–45 DAP), the relative level of L3 gene transcription decreased further to nearly 12-fold less than the peak level. However, this latter decrease in L3 gene transcription is due in part to the nonspecific 3–4-fold decrease in overall level of transcription in the older embryos. The pattern of L3 gene transcription during seed development parallels that of steady-state L3 mRNA levels shown in Fig. 4*A*. This result suggests that the level of L3 mRNA in developing embryos is regulated, at least in part, by the level at which the L3 gene is transcribed.

Effect of Exogenous Abscisic Acid on L3 Gene Expression—The plant growth regulator abscisic acid has been implicated in the regulation of gene expression during seed development (2, 25). In particular, abscisic acid is thought to be important in maintaining embryogeny and in suppressing germination. When mature maize seeds were allowed to imbibe in the presence of 10^{-4} M abscisic acid, less than 2% of the seeds germinated. The percent germination increased to 50% at 10^{-5} M abscisic acid and 98% at 10^{-6} M abscisic acid. To determine if abscisic acid also affects L3 gene expression, seeds were allowed to imbibe in the presence of various concentrations of the exogenous abscisic acid. The steady-state levels of L3 mRNA were measured by RNA blot analysis. Seeds imbibed in water (or in 10^{-6} M abscisic acid, data not shown) germinated normally, and L3 mRNA was present at

12 and 24 h post-imbibition and then declined to an undetectable level at 48 h and was not detected at any later time (Fig. 6; see also Fig. 3). In contrast, most of the seeds imbibed in 10^{-4} M abscisic acid failed to germinate, and the normal development decline in the L3 mRNA level at 48 h post-imbibition was prevented (Fig. 6). At 10^{-5} M abscisic acid, only about one-half of the seeds germinated. The level of L3 mRNA declined normally in those that germinated, but failed to decline in the remaining seeds that did not germinate (data not shown). Thus, seed germination is coupled to the developmental decline in L3 gene expression. Furthermore, exogenous abscisic acid, either directly or indirectly, suppresses germination and extends the developmental time course of L3 gene expression.

We examined the role of L3 gene transcription in regulating the extended period of L3 mRNA accumulation in abscisic acid-imbibed seeds. The relative level of L3 gene transcription in maize embryos from mature seeds imbibed in the presence or absence of abscisic acid was determined by run-off transcription in isolated nuclei. Seeds were imbibed in water or 10^{-4} M abscisic acid, and embryos were isolated 48 h after imbibition. Nuclei were isolated and incubated in the presence of [^{32}P]UTP. The total incorporation of [^{32}P]UTP per unit of DNA in the transcription reaction was approximately the same for nuclei with or without abscisic acid treatment. Fig. 7 shows that the proportion of transcriptional activity devoted to L3 gene transcription was at least 10-fold higher in embryos isolated from seeds imbibed in abscisic acid than in those from seeds imbibed in water. In contrast, the proportion of transcriptional activity devoted to the alcohol dehydrogenase gene was nearly the same in water- or abscisic acid-imbibed seeds. Little or no nonspecific hybridization to plasmid DNA was noted. These data indicate that the elevated level of L3 mRNA in abscisic acid-imbibed seeds is due, at least in part, to the continued transcription of the L3 gene.

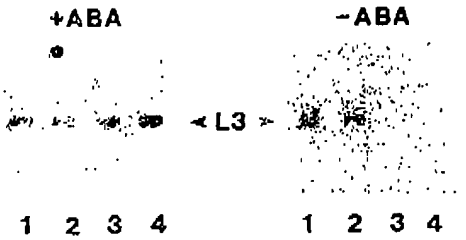

FIG. 6. Effect of abscisic acid on the steady-state level of L3 mRNA in maize embryos during seed germination. Total RNA was isolated from scutella at the following times after imbibition of mature seed in either 10^{-4} M abscisic acid (*+ABA*) or water (*−ABA*) and examined by RNA blot analysis: *lane 1*, 12 h; *lane 2*, 24 h; *lane 3*, 48 h; *lane 4*, 72 h. Equal amounts of RNA were run in each lane of an agarose gel, and all the samples were run on the same gel and blotted and hybridized together. The position of L3 mRNA is indicated.

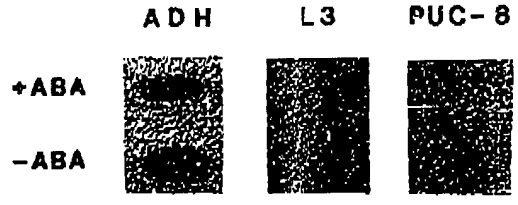

FIG. 7. Effect of abscisic acid on transcription of L3 gene in isolated nuclei from maize embryos during seed germination. Seeds were imbibed in the presence of 10^{-4} M abscisic acid (*+ABA*) or water (*−ABA*) for 48 h. Embryos were removed, and their nuclei were isolated and incubated in the presence of [^{32}P]UTP under conditions which allowed the elongation of nascent transcripts. Equal number of counts incorporated into the run-off transcripts were hybridized to alcohol dehydrogenase (*ADH*), L3, or nonrecombinant plasmid DNA (*PUC-8*) bound to paper.

DISCUSSION

L3 protein accumulates in the embryo and the aleurone layer of developing maize seed. Northern analyses indicate that L3 mRNA also accumulates to a high level in the embryo, suggesting that L3 protein is synthesized and accumulates in the same cells. The major triacylglycerol reserves in the maize seed have been localized to the embryo and aleurone tissue by histochemical staining (26). These findings suggest that L3 is unique to lipid bodies and that the lipid bodies in scutellum, embryonic axes, and aleurone are probably biochemically similar.

Our experimental results indicate that the L3 gene is a member of the set of genes whose expression is increased by abscisic acid. Like many other plant genes whose expression is enhanced by abscisic acid (27–30), the L3 gene is expressed only in the embryo and aleurone tissue during seed development. In several cases, abscisic acid-enhanced protein accumulation has been correlated with increased levels of the associated mRNA (for example, see Refs. 31–34). However, it is not known if the increased levels of mRNA are due to an effect of abscisic acid on gene transcription, RNA stability, and/or RNA processing. Our experimental results show that exogenously applied abscisic acid increases the steady-state level of L3 mRNA by increasing the level of L3 gene transcription.

L3 gene expression is not only tissue-specific, but also developmentally regulated. The steady-state level of L3 mRNA is modulated during seed development, increasing in early stages and then decreasing as the seed matures. This modulation in L3 mRNA levels is controlled, at least in part, by changes in the level at which the L3 gene is transcribed. Changes in the stability or processing of L3 mRNA may also be involved, and this was not ruled out. It is possible that the specific developmental changes in L3 gene transcription are regulated by the endogenous abscisic acid concentration in the embryo. Interestingly, the concentration of endogenous abscisic acid in the maize embryo has recently been shown to peak and decline with a time course similar to that of L3 mRNA accumulation (35).

The mechanisms which control L3 gene expression are different from those regulating the expression of zeins, the major storage proteins in maize. The zeins are encoded by large multigene families (1), whereas L3 protein is encoded by only one or a few genes. In addition, the zeins and L3 protein are expressed in different tissues and with different developmental time courses. The zeins are found only in the central endosperm (36), whereas L3 protein is confined to the embryo and aleurone tissues. Both the zeins and L3 protein are expressed at their highest level during early seed development. However, zein gene expression ceases as the seed matures and the endosperm dies. In contrast, L3 gene expression continues at a low level through the first day of germination. Our data show that the level of L3 gene transcription is increased in the presence of exogenous abscisic acid. Abscisic acid has not been implicated in the regulation of zein gene expression; rather, a tremendous endoreduplication of the nuclear DNA in the central endosperm may play a role in regulating the developmental accumulation of zeins (1, 37). Thus, although both L3 and zein genes are involved in seed storage metabolism, they differ with regard to gene copy

number, tissue specificity, time course of expression, and mechanisms of gene regulation.

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Molecular Characterization of the Major Maize Embryo Globulin Encoded by the *Glb1* Gene[1]

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ABSTRACT

One of the most abundant proteins in maize (*Zea mays* L.) embryos is the molecular weight 63,000 globulin encoded by the *Glb1* gene. To obtain DNA clones corresponding to RNA from developing maize embryos was constructed in a lambda expression vector and screened with antibodies specific for *Glb1*-encoded proteins. Here we report the complete nucleotide sequence, as determined from two overlapping clones, of pcGlb1S, a 2009 base pair clone containing the entire translated region of *Glb1*. The deduced amino acid sequence of pcGlb1S shows similarities to 7S-type seed storage proteins of wheat and legumes. Southern blot analysis of maize DNA confirms previous genetic studies which had indicated the presence of a single copy of *Glb1* per haploid genome. Northern blot analysis indicates that *Glb1* transcripts are present throughout most of embryo development and that expression of this gene is limited to seed tissues. Embryos homozygous for a *Glb1* null allele, in which *Glb1*-encoded proteins are not detectable, contain low levels of *Glb1* transcripts which are a different size from those encoded by functional alleles. This suggests that the defect in the null allele is at the level of gene transcription or RNA processing.

Maize embryos contain large amounts of saline-soluble, water-insoluble proteins called globulins. The major globulin component, a mol wt 63,000 protein designated GLB1 (formerly PROT) (10), is one of the most abundant proteins in mature embryos. GLB1 has no known enzymatic function and is currently believed to serve as a storage protein (8). Genetic analysis of GLB1 variants indicated that this protein is encoded by a single gene, *Glb1* (for *Globulin-1*), on the long arm of chromosome 1 (22). Several alleles of this gene, including a CRM^- null,[2] have been described (17, 22). Allelic polymorphism of *Glb1*-encoded proteins is observed as a function of mobility in SDS-PAGE. The three most commonly occuring *Glb1* alleles have the designations *L*, *I*, and *S*, for *Large*, *Intermediate*, and *Small* proteins, respectively. By convention, the protein product of each allele is indicated in the upper case (*e.g. Glb1-S* encodes GLB1-S). Pulse-chase radiolabeling and *in vitro* translation experiments have indicated that at least three protein-processing steps occur in the formation of the mature protein from the primary translation product (10). The final processing step is controlled by the unlinked gene *Mep* (chromosome 5, near *Pr*), and embryos homozygous for the recessive *mep* allele accumulate the processing intermediate GLB1′ (10, 22). We have recently reported on additional characterization of maize embryo globulins (8).

Here we report the isolation and characterization of a full-length[3] cDNA clone, pcGlb1S,[4] corresponding to the S allele of the *Glb1* gene. Using this clone as a probe, we have determined that expression of the gene appears to be limited to seed tissues and that embryos homozygous for the null allele contain small amounts of transcript corresponding to *Glb1*.

MATERIALS AND METHODS

Materials

Embryos homozygous for the *Glb1-L* and *Glb1-S* alleles were obtained from field-grown plants of the maize (*Zea mays* L.) inbred lines W64A and Va26, respectively, as previously described (8). The *Glb1-O* null allele was originally identified in a Black Beauty popcorn line (22). Nitrocellulose and Magnagraph nylon membranes were obtained from Micron Separations, Inc. (Westboro, MA). Random priming kits, a cDNA synthesis kit, *Eco*RI, T4 DNA ligase, S1 nuclease, and protein and RNA mol wt standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD). LambdaZAP vector arms, Gigapack packaging extracts, and exonuclease III/mung bean nuclease deletion kits were from Stratagene (La Jolla, CA). [α-^{32}P]-labeled dATP (3000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Oligo(dT)-cellulose (type 7) was from Pharmacia, Inc. (Piscataway, NJ).

Preparation of GLB1-Specific Antiserum

GLB1-S was fractionated from whole globulin by cryoprecipitation (8) and stored at 4°C as a suspension in distilled water at a concentration of 2 mg/mL. A volume of the suspension equivalent to 600 μg protein was emulsified with an equal volume of Freund's complete adjuvant and injected

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[2] Abbreviations: CRM, cross-reacting material; DAP, days after pollination; ds, double-stranded.

[3] The term full-length as used here indicates that the cDNA clone contains the entire translated region of the gene.

[4] The GenBank accession number for pcGlb1S is M24845.

ubcutaneously into multiple sites of a 10-week old New Zealand White rabbit. A second inoculation of 300 μg GLB1- in Freund's incomplete adjuvant was administered 2 weeks ater. Blood was collected from the marginal ear vein 3 weeks ifter the secondary inoculation and serum was prepared from he blood by standard methods.

Protein Extraction and Immunoblot Analysis

For analysis of total proteins from imbibed mature embryos 8), kernels were soaked overnight in water and embryos excised from the softened kernels with the aid of a sharpened spatula. For developmental analyses, field-grown ears were transported to the laboratory and the crowns of the kernels were sliced off with a sharp knife or scalpel blade. Embryos were removed from the kernels with a small spatula, immediately frozen in liquid nitrogen, and stored at −70°C until needed. Individual mature embryos were macerated in 1 mL SDS sample buffer (2% [w/v] SDS, 50 mM Tris [pH 6.8] 5% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol) at a ratio of 50 mg/mL (fresh weight) directly in 1.5 mL microcentrifuge tubes with the aid of a fitted pellet pestle (Kontes Scientific Glassware, Morton Grove, IL). Extracts were heated to 100°C for 2 min, centrifuged at 13,500*g* for 5 min, and immediately subjected to SDS-PAGE as previously described (8). Five μL of each sample was applied to a 12.5% polyacrylamide gel and subsequently electroblotted onto nitrocellulose by using a semidry blotting apparatus (PolyBlot; American Bionetics, Hayward, CA) according to the manufacturer's specifications. Immunoblot analysis, in which GLB1-specific antiserum was used at 1:2000 dilution, was performed as previously described for analysis of maize zeins (13).

Nucleic Acid Isolation and Gel Blot Analysis

Total RNA was isolated from frozen tissue by using the guanidine-HCl method described by Cox (3). For Northern blot analysis, 10 μg of total RNA was subjected to electrophoresis in formaldehyde agarose gels and transferred to either nitrocellulose or nylon (Magnagraph) membranes as described by Selden (23). Hybridization conditions were as previously described (9).

Isolation of maize nuclear DNA from unfertilized ears and Southern blot analysis were performed as previously described (9) except that Magnagraph membranes were used as the transfer medium. Samples for gene copy-number reconstructions contained *Eco*RI-cleaved DNA from a genomic clone corresponding to *Glb1-L* (our unpublished data). For both RNA and DNA blots, the transferred nucleic acids were UV-linked to the membrane by using a Stratalinker 1800 apparatus (Stratagene).

For use in hybridizations, the cDNA insert was isolated from the plasmid clone by *Eco*RI digestion, separation on a 1% agarose gel, and binding of the fragment to NA-45 paper (Schleicher and Schuell, Keene, NH) as recommended by the manufacturer. The isolated insert was labeled with [α-^{32}P] dATP by using a commercial random priming kit (BRL).

Construction and Screening of an Embryo-Specific cDNA Library

To obtain cDNA clones corresponding to *Glb1*, a library of cDNA sequences was prepared in a bacteriophage λ-expression vector. Total RNA was prepared from 27 DAP embryos of the maize inbred line Va26 *(Glb1-S/S)*. Polyadenylated RNA was fractionated from total RNA by oligo(dT)-cellulose chromatography (1). First and second strand cDNA synthesis was performed by using a commercial cDNA synthesis kit based on the RNase H procedure of Gubler and Hoffman (4). The ds cDNA was protected from *Eco*RI cleavage by treatment with *Eco*RI methylase, and *Eco*RI oligonucleotide linkers (both from New England Biolabs, Beverly, MA) were added to the ends of the ds cDNA with T4 DNA ligase. Subsequent to linker cleavage with *Eco*RI, excess linkers were removed by passage through a NACS Prepac column (BRL) as described by the manufacturer. The ds cDNA was ligated to the arms of the expression vector LambdaZAP, packaged by using the Gigapack system, and the resultant recombinant phage was used to infect the *Escherichia coli* host BB4. The primary library consisted of approximately 500,000 clones which were obtained from an estimated 0.1 μg of ds cDNA.

The initial plating of the library was screened by preparing nitrocellulose replicas of the plates by the plaque-lift technique as described by Huynh *et al.* (7). Filters were agitated in a solution of TTBS/1% (w/v) nonfat dry milk (TTBS: 50 mM Tris [pH 7.4] 0.15% [v/v] Tween-20, 140 mM NaCl) for 1 h, then transferred to a solution of GLB1-specific antibody (1:500 dilution in TTBS) and agitated overnight. Immunoreactive clones were detected as for the protein immunoblots. Plaques were picked, plated, and subjected to three additional rounds of screening. Of 10 initial plaque picks, all continued to yield positive reactions after the final screen. The cDNA inserts were excised from LambdaZAP as recombinant pBluescript SK(-) plasmids according to the manufacturer's protocols. Rescreening of the library with a radiolabeled probe was essentially as described by Huynh *et al.* (7).

DNA Sequencing

For nucleotide sequence analysis, the cDNA inserts in pBluescript were subcloned into M13 mp18 and mp19 (30) to obtain inserts in opposite orientations. Overlapping unidirectional deletions corresponding to either strand were prepared from the appropriate M13 clone RF by using a commercial exonuclease III/mung bean nuclease deletion kit (Stratagene). The deletions were sized by using a rapid S1 nuclease procedure (16). Dideoxynucleotide sequencing (21) of single-stranded templates with a modified T7 DNA polymerase (Sequenase) was performed by using a commercial sequencing kit (United States Biochemical Corp., Cleveland, OH). The deoxyguanine triphosphate (dGTP) analog 7-deaza dGTP was used to resolve GC compressions. Analysis of DNA sequences was performed on an IBM PC AT with either IBI/Pustell Sequence Analysis software (International Biotechnologies Inc., New Haven, CT) or DNAStar programs (DNAStar, Inc., Madison, WI).

SULTS

Specific Antibodies

Characterizat -specific polyclonal antibodies was oblot of proteins from individual composition (Fig. 1). Lanes 1 and The spe bryos of the inbred lines W64A and determi zygous for the *Glb1-L* and *Glb1-S* embry major proteins recognized by the 2 of L and GLB1-S and their respective GLB1'-L and GLB1'-S (10). There other polypeptide bands of lower mol with the antiserum. Since these are homozygous for the *Glb1-O* null allele ferred that these low mol wt polypeptides Glb1-encoded proteins. Such polypeptides *lb1-L/O* and *Glb1-S/O* embryos (Fig. 1, pectively), but at levels lower than those -L/L or *S/S* embryos. This is apparently ce of only a single functional *Glb1* allele in s. We therefore concluded that the antiserum *Glb1*-encoded proteins and was appropriate ning a cDNA expression library.

Characterization of pcGlb1S

nary experiments we observed that embryos of inbred line Va26 contained large amounts of GLB1- otein. We therefore chose this material as a source of RNA for production of a cDNA library as described above. A screen of 250,000 clones yielded 10 immunoreactive clones with inserts ranging in size from 700 to 1800 bp. Southern blot analysis revealed that all of these clones hybridized with a radiolabeled probe prepared from the longest insert.

The 1800 bp clone, designated pcA1, was chosen for further characterization and subjected to nucleotide sequence analysis. A 300 bp *Eco*RI/*Bst*EII restriction fragment from the 5′ region of this clone was used as a radiolabeled probe to rescreen the cDNA library for a full length cDNA clone, which we have designated pcGlb1S. The 5′ region of the full length clone was sequenced to provide 55 nucleotides of overlap with the original 1800 bp clone pcA1. The nucleotide sequences of the two clones were identical in this region of overlap. Because of the high G + C content (66%) of these clones, it was necessary to use the dGTP analog deaza dGTP to minimize GC compressions and obtain unambiguous sequence data. The nucleotide sequence was determined from both strands for the appropriate regions of each clone.

The combined sequence of the two cDNA clones and the deduced amino acid sequence are shown in Figure 2. The sequence surrounding the presumed initiator methionine corresponds to a relaxed consensus (27) of Kozak's rule, ANNATGG (11, 14). An AT-rich region between nucleotide positions +1937 and +1949 is considered to be a potential polyadenylation signal and contains the consensus sequence AATAAA (18). The nucleotide sequence predicts a polypeptide of 573 amino acids with a calculated mol wt of 65,025 D. From SDS-PAGE analysis of immunoprecipitated *in vitro* translation products, the mol wt for the primary translation product of *Glb1-S* has been estimated as 67,600 D (10). The mol wt calculated from the deduced amino acid sequence is 2,575 D lower than the experimentally determined estimate. We do not consider this discrepancy to be significant since it is not uncommon for proteins to exhibit anomalous migration in SDS-PAGE (2). The presence of a region of deduced amino acid sequence (*underlined* in Fig. 2) identical to the N-terminal region of experimentally determined GLB1-S protein sequence (8) confirms that these cDNA clones correspond to *Glb1*. Previous studies demonstrated that GLB1′ and GLB1 differ at only one terminus (10). The present sequence analysis indicates that the final processing of GLB1′ to GLB1, controlled by the *Mep* gene (22), is localized to the N-terminal portion of GLB1′. In GLB1′-S, this cleavage probably occurs between the aspartate and glutamate residues at positions 86 and 87 (Fig 2, arrowhead). The single internal methionine at amino acid position 355 (Fig. 2, boxed) is consistent with previous cyanogen bromide cleavage analysis (10) which yielded two cleavage fragments corresponding in size to those predicted from this sequence analysis.

The amino acid composition deduced from the pcGlb1S sequence in the region corresponding to GLB1-S (Table I) is in close agreement with that obtained from direct analysis of the GLB1-S protein (8). The protein contains high amounts of glutamate, arginine, serine, and glycine, and low amounts of cysteine, methionine, and tryptophan.

A hydropathy plot (12) of the deduced protein sequence is depicted in Figure 3. The sequence is extremely hydrophilic with the exception of a strongly hydrophobic region corre-

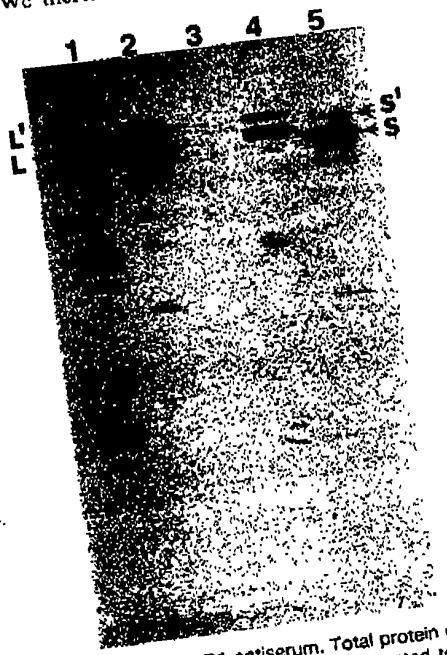

Figure 1. Specificty of GLB1 antiserum. Total protein extracts from embryos of varying *Glb1* genotypes were subjected to immunoblot analysis as described in "Materials and Methods." Lane 1, *Glb1-L/L* (W64A); lane 2, *Glb1-S/S* (Va26); lane 3, *Glb1-O/O*; lane 4, *Glb1-L/O* (W64A x *Glb1-O/O*); lane 5, *Glb1-S/O* (Va 26 x *Glb1-O/O*).

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-36 CGCACACACCCGAGCATATCACAGTGACACTACACG ATG GTG AGC GCC AGA ATC GTT GTC CTC CTC GCC GTC CTC CTA TGC GCT GCC GCC GCA GTC GCG
                                          M  V  S  A  R  I  V  V  L  L  A  V  L  L  C  A  A  A  A  V  A

+64 TCG TCC TGG GAG GAC GAC AAC CAC CAC CAC CAC GGG GGC CAC AAG TCC GGG CGA TGC GTG CGG CGG TGC GAG GAC CGG CCC TGG CAC CAG
     S  S  W  E  D  D  N  H  H  H  H  G  G  H  K  S  G  R  C  V  R  R  C  E  D  R  P  W  H  Q

+154 CGC CCC CGG TGC CTG GAG CAG TGC AGG GAG GAG GAG CGG GAG AAG CGG CAA GAG CGG AGC AGG CAC GAG GCC GAC GAC CGC AGC GGC GAG
      R  P  R  C  L  E  Q  C  R  E  E  E  R  E  K  R  Q  E  R  S  R  H  E  A  D  D  R  S  G  E

+244 GGC TCG TCG GAG GAT GAG CGC GAG CGC GAG CAG GAG AAG GAG GAG AAG CAG AAG GAC CGG CGG CCG TAC GTG TTC GAC CGG CGC AGC TTT
      G  S  S  E  D  E  R  E  R  E  Q  E  K  E  E  K  Q  K  D  R  R  P  Y  V  F  D  R  R  S  F

+334 CGT CGC GTG GTC CGG AGC GAG CAG GGG TCC CTG AGG GTG CTC CGG CCG TTC GAC GAG GTG TCC AGG CTC CTC CGC GGC ATC CGG GAC TAC
      R  R  V  V  R  S  E  Q  G  S  L  R  V  L  R  P  F  D  E  V  S  R  L  L  R  G  I  R  D  Y

+424 CGC GTG GCG GTC CTG GAG GCG AAC CCG CGC TCG TTC GTG GTG CCC AGC CAC ACC GAC GCG CAC TGC ATC GGC TAC GTG GCG GAA GGC GAG
      R  V  A  V  L  E  A  N  P  R  S  F  V  V  P  S  H  T  D  A  H  C  I  G  Y  V  A  E  G  E

+514 GGA GTG GTG ACG ACG ATC GAG AAC GGC GAG AGG CGG TCG TAC ACC ATC AAG CAA GGC CAC GTC TTC GTG GCG CCG GCC GGG GCG GTC ACC
      G  V  V  T  T  I  E  N  G  E  R  R  S  Y  T  I  K  Q  G  H  V  F  V  A  P  A  G  A  V  T

+604 TAC CTG GCC AAC ACC GAC GGC CGG AAG AAA CTG GTC ATC ACC AAG ATC CTC CAT ACC ATC TCC GTG CCT GGC GAG TTC CAG TTC TTC TTC
      Y  L  A  N  T  D  G  R  K  K  L  V  I  T  K  I  L  H  T  I  S  V  P  G  E  F  Q  F  F  F

+694 GGC CCC GGC GGG AGG AAC CCG GAA TCG TTC CTG TCG AGC TTC AGC AAG AGC ATC CAG AGA GCT GCG TAC AAG ACC TCG AGC GAC CGG CTG
      G  P  G  G  R  N  P  E  S  F  L  S  S  F  S  K  S  I  Q  R  A  A  Y  K  T  S  S  D  R  L

+784 GAG AGG CTG TTC GGG AGG CAT GGG CAG GAC AAG GGG ATC ATC GTG CGT GCC ACG GAG GAG CAG ACC CGC GAG CTG CGG CGC CAC GCC TCG
      E  R  L  F  G  R  H  G  Q  D  K  G  I  I  V  R  A  T  E  E  Q  T  R  E  L  R  R  H  A  S

+874 GAG GGC GGC CAC GGC CCG CAC TGG CCC CTG CCG CCG TTC GGC GAG TCG CGC GGC CCC TAC AGC CTC CTG GAC CAG CGG CCC AGC ATC GCC
      E  G  G  H  G  P  H  W  P  L  P  P  F  G  E  S  R  G  P  Y  S  L  L  D  Q  R  P  S  I  A

+964 AAC CAG CAC GGG CAG CTC TAC GAG GCC GAC GCG CGC AGC TTC CAC GAC CTC GCC GAG CAC GAC GTC AGC GTC TCC TTC GCC AAC ATC ACC
      N  Q  H  G  Q  L  Y  E  A  D  A  R  S  F  H  D  L  A  E  H  D  V  S  V  S  F  A  N  I  T

+1054 GCG GGG TCC ATG AGC GCG CCA TTG TAC AAC ACC CGT TCG TTC AAG ATC GCC TAC GTG CCG AAC GGC AAG GGC TAC GCC GAG ATC GTG TGC
       A  G  S  M  S  A  P  L  Y  N  T  R  S  F  K  I  A  Y  V  P  N  G  K  G  Y  A  E  I  V  C

+1144 CCG CAC CGC CAG TCG CAG GGC GGC GAG AGC GAG CGC GAG CGC GGC AAG GGC AGG AGG AGC GAA GAA GAA GAA GAA TCG TCT GAG GAG CAG
       P  H  R  Q  S  Q  G  G  E  S  E  R  E  R  G  K  G  R  R  S  E  E  E  E  E  S  S  E  E  Q

+1234 GAG GAA GTC GGG CAG GGG TAC CAC ACC ATC CGG GCG CGG CTG TCA CCG GGC ACG GCG TTC GTG GTG CCC GCG GGC CAC CCG TTC GTC GCG
       E  E  V  G  Q  G  Y  H  T  I  R  A  R  L  S  P  G  T  A  F  V  V  P  A  G  H  P  F  V  A

+1324 GTG GCG TCC CGG GAC AGC AAC CTC CAG ATC GTG TGC TTC GAG GTC CAC GCC GAC AGG AAC GAG AAG GTG TTC CTG GCC GGC GCC GAC AAC
       V  A  S  R  D  S  N  L  Q  I  V  C  F  E  V  H  A  D  R  N  E  K  V  F  L  A  G  A  D  N

+1414 GTG CTG CAG AAG CTC GAC CGG GTC GCC AAG GCG CTG TCA TTC GGC TCC AAG GCG GAG GAG GTG GAC GAG GTG CTC GGC TCG CGG CGC GAG
       V  L  Q  K  L  D  R  V  A  K  A  L  S  F  A  S  K  A  E  E  V  D  E  V  L  G  S  R  R  E

+1504 AAG GGG TTC CTT CCT GGC CCC AAG GAG AGC GGC GGC CAC GAG GAG CGG GAG CAG GAG GAG GAG GAA CGC GAA GAA CGC CAC GGC GGG CGT
       K  G  F  L  P  G  P  K  E  S  G  G  H  E  E  R  E  Q  E  E  E  E  R  E  E  R  H  G  G  R

+1594 GGG GAG AGG GAA CGC CAC GGA CGT GAG GAG CGG GAG AAA GAG GAG GAG GAA CGC GAA GGA CGC CAC GGC CGC GGG CGC CGC GAG GAA GTG
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+1684 GCG GAG ACG CTC CTG AGG ATG GTG ACC GCC AGG ATG TGA GGCCGGCCGTGCTCGCCAAAACGAGCAGGAAGCAACGAGAGGGTGGCGCGCGACCGACGTGCGTACGTA
       A  E  T  L  L  R  M  V  T  A  R  M  *

+1792 GCATGAGCCTGAGTGGAGACGTTGGACGTGTATGTATATACCTCTCTGCGTGTTAACTATGTACGTAAGCGGCAGGCAGTGCAATAAGTGTGGCTCTGTAGTATGTACGTGCGGGTACGAT

+1913 GCTGTAAGCTACTGAGGCAAGTCCATAAATAAATAATGACACGTGCGTGTTCTATAAAAAA  +1973
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Figure 2. Complete nucleotide and deduced amino acid sequence of pcGlb1S. The sequence from nucleotide positions −36 to +189 was determined directly from pcGlb1S, and the sequence from positions +190 to +1973 was determined from pcA1. The region of overlap described in the text extends from position +190 to +245. The open reading frame extends from nucleotide positions +1 to +1719. The TGA termination codon is indicated by an asterisk. The deduced amino acid sequence from positions +259 to +300 (amino acids 87–100, *underlined*) corresponds to the N-terminal amino acid sequence determined directly from GLB1-S (8). An AT-rich region at positions +1937 to +1949 *(underlined)* is considered to be a potential polyadenylation site. Proposed sites for potential signal peptide cleavage are indicated by the two arrows, and the proposed site for *Mep*-catalyzed cleavage is indicated by an arrowhead. The internal methionine residue detected by CNBr cleavage (10) is boxed.

Table I. *Deduced Amino Acid Compositions of Glb1-S Gene Products*

Values were determined from the pcGlb1S nucleotide sequence in the indicated regions

	Amino Acid Position			
	87-573[a]		1-573[b]	
	mol%	No. aa	mol%	No. aa
Glu	13.35	65	13.26	76
Arg	12.11	59	12.57	72
Gly	9.45	46	8.90	51
Ser	7.60	37	7.85	45
Val	7.39	36	7.53	42
Ala	7.19	35	7.50	43
Leu	5.75	28	5.76	33
Pro	4.52	22	4.19	24
Phe	4.52	22	3.84	22
Lys	4.11	20	3.84	22
His	3.90	19	4.54	26
Gln	3.70	18	3.66	21
Asp	3.49	17	4.01	23
Thr	3.49	17	2.97	17
Ile	3.29	16	2.97	17
Tyr	2.46	12	2.09	12
Asn	2.26	11	2.09	12
Trp	0.21	1	0.52	3
Cys	0.62	3	1.40	8
Met	0.62	3	0.70	4
		487		573

[a] Corresponds to GLB1-S. [b] Corresponds to primary translation product.

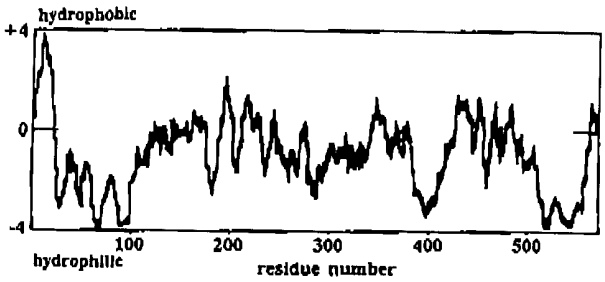

Figure 3. Hydropathy plot of deduced protein sequence of GLB1-S. This plot was generated by using the algorithm of Kyte and Doolittle (12), as supplied with DNAStar sequence analysis programs, using a window of seven amino acids.

sponding to the 21 N-terminal amino acids. This hydrophobic region is similar to the general characteristics of transmembrane peptide signal sequences (28). In particular, this region has a high degree of homology with the presumed signal sequence of aleurainc, a secreted thiol protease from barley aleurone (20). The two most likely sites for cleavage of the presumed signal sequence (Fig. 2, arrows) were predicted by the weighted matrix method of von Heijne (26). To conform with standard designations for processing intermediates which contain signal sequences, the primary translation product will subsequently be referred to as preproGLB1′ and the short-lived *in vivo* precursor, previously referred to as preGLB1′ (10), will be designated proGLB1′.

Genomic Representation of *Glb1*

Genetic analysis demonstrated that GLB1 is encoded by a single gene (22). In Southern blot analysis of maize DNA from the inbred line W64A *(Glb1-L/L)*, pcGlb1S hybridized to a single *Eco*RI fragment of 3.4 kb (Fig. 4, lane 1) as would be expected for a single gene since there are no *Eco*RI sites within the clone. Gene copy-number reconstruction analysis confirms the presence of a single copy of *Glb1* per haploid genome (Fig. 4). An identically sized *Glb1*-specific *Eco*RI fragment is present in DNA from plants homozygous for the

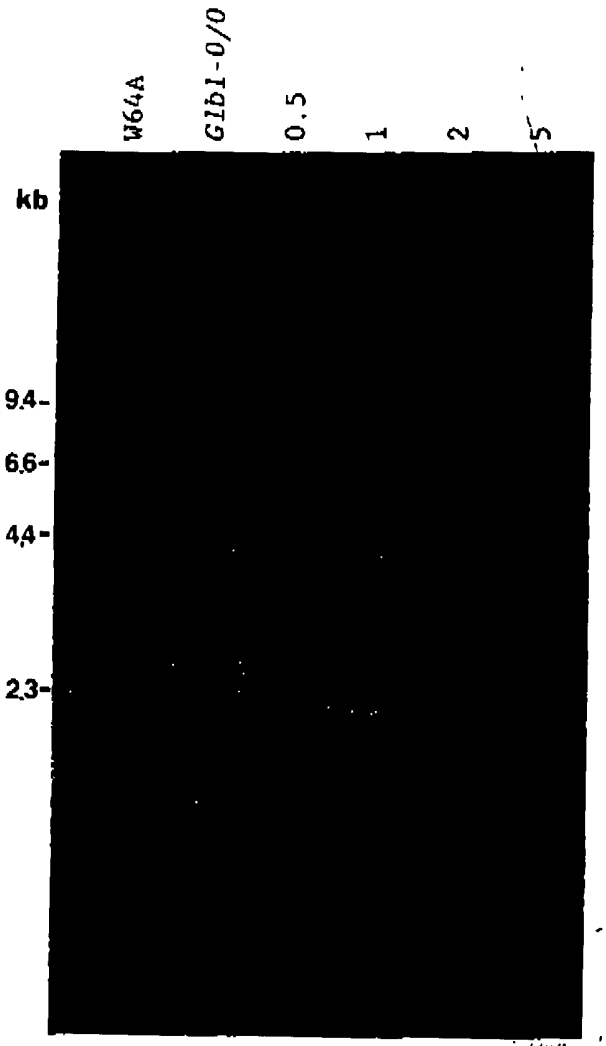

Figure 4. Southern blot analysis of maize DNA from W64A *(Glb1-L/L)* and *Glb1-O/O* plants. The first two lanes each contained 5.3 μg *Eco*RI-cleaved DNA from either of the two genotypes, as indicated. This amount of DNA corresponds to 1 x 10^6 haploid genomes. Gene copy-number reconstruction lanes contained *Eco*RI-cleaved DNA from a *Glb1-L* genomic clone (our unpublished data) corresponding to 0.5 x 10^6, 1 x 10^6, 2 x 10^6, or 5 x 10^6 copies of the cloned fragment, as indicated. The blot was probed with the radiolabeled cDNA insert from pcGlb1S.

ull allele (Fig. 4, lane 2). This indicates that the lack of GLB1 rotein in the null is not due to simple deletion of the gene. ı addition, no differences in the hybridization pattern in *lam*HI and *Hind*III digests were observed for the two geno- ypes (data not shown).

lorthern Blot Analysis of Normal and *Glb1-O/O* Embryos

Embryos homozygous for the *Glb1* null allele lack detectble GLB1 protein (22) and translatable *Glb1* mRNA as etermined by immunoprecipitation of *in vitro* translation roducts (10). To determine if *Glb1* transcripts are present in *ilb1* null embryos, total RNA from *Glb1-L/L* and *Glb1-O/*) embryos at 24 and 27 DAP was subjected to Northern blot nalysis in which the ^{32}P-labeled insert from pcGlb1S was ısed as probe (Fig. 5). As expected, a single band in the *Glb1-* *L/L* samples exhibited strong hybridization with the probe Fig. 5, lanes 1 and 3). A faint band of slightly larger size was letected in the *Glb1-O/O* samples at both 24 and 27 DAP Fig. 5, lanes 2 and 4). The apparent size of the transcript in *Glb1-L/L* embryos is 2.4 kb and that of the *Glb1-O/O* embryos is 2.5 kb. There was no detectable size difference between transcripts from embryos of *Glb1-L/L* and *Glb1-S/* S inbred lines, and only a single band was observed in RNA rom *Glb1-L/S* embryos (data not shown).

Developmental Accumulation and Seed-Specificity of *Glb1* Transcripts

The accumulation of *Glb1* transcripts during embryo development is depicted in Figure 6. Total RNA from *Glb1-L/* *L* embryos at 18 to 42 DAP was subjected to Northern blot analysis in which the pcGlb1S insert was used as radiolabeled probe. The level of Glb1-specific transcripts increased from

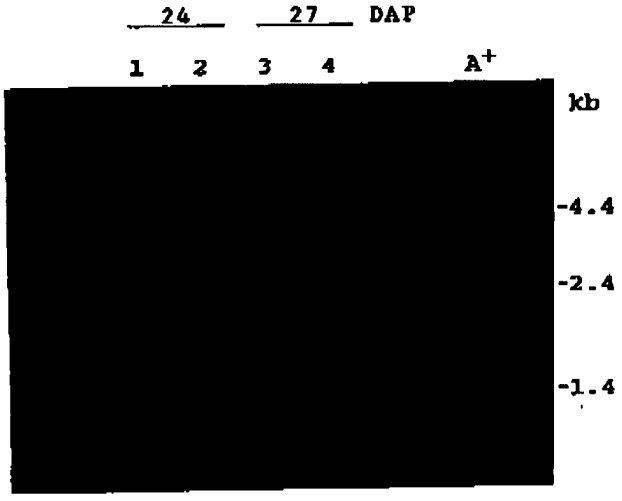

Figure 5. Northern blot analysis of RNA from embryos of different *Glb1* genotypes. Ten μg of total RNA from *Glb1-L/L* (W64A; lanes 1 and 3) or *Glb1-O/O* (lanes 2 and 4) embryos at 24 or 27 DAP, as indicated, or 1 μg of polyadenylated RNA from 27 DAP *Glb1-S/S* (Va26) embryos (A$^+$), was subjected to Northern blot analysis in which the pcGlb1S insert was used as probe. Positions of RNA size markers (BRL) are as indicated.

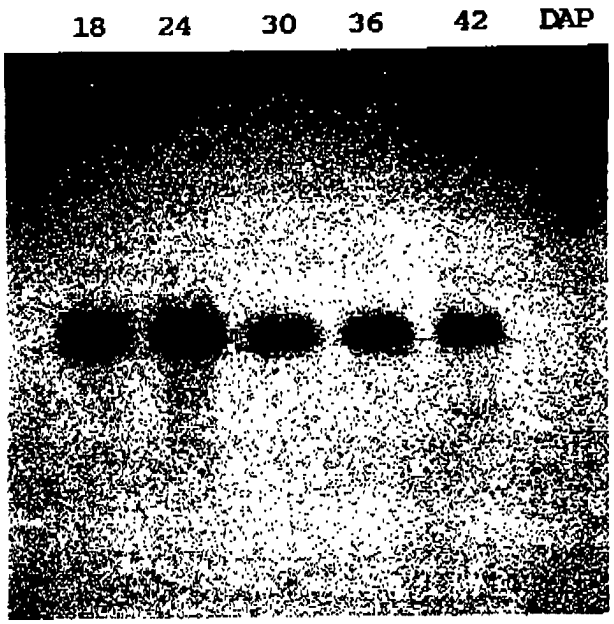

Figure 6. Developmental accumulation of *Glb1* transcripts. Total RNA (10 μg/lane) from developing *Glb1-L/L* embryos (18–42 DAP, as indicated) was subjected to Northern blot analysis in which the radiolabeled pcGlb1S cDNA insert was used as probe.

18 to 24 DAP, then decreased by 30 DAP. The 30 DAP level was maintained through 42 DAP.

Glb1 expression was also investigated in plant tissues other than the embryo. Total RNA samples were obtained from *Glb1-L/L* embryos, endosperm, unfertilized ears, immature tassels, and 7-d old seedlings. *Glb1*-Specific transcripts were detected in the endosperm but at a lower level than that found in the embryo (Fig. 7, lanes 1 and 2). *Glb1* transcripts were not detected in any of the other tissues examined. Identical results were obtained when similar tissues from the *Glb1-S/S* inbred line were subjected to the same analysis (data not shown).

DISCUSSION

To further characterize the maize *Glb1* gene, which encodes an abundant protein in the embryo, we have isolated and determined the nucleotide sequence of a cDNA clone, pcGlb1S, corresponding to this locus. Nucleotide sequence analysis of this 2009 bp clone reveals an open reading frame corresponding to 573 amino acids. The deduced amino acid sequence and composition are consistent with information obtained for GLB1-S (8). The nucleotide sequence contains consensus sequences for the initiator methionine and a 3′ polyadenylation sequence. The pcGlb1S sequence has a high G+C content of 66%; similar high G+C contents have been observed for genes encoding other seed proteins (20).

Sequence analysis indicates that the protein product of the *Glb1* gene appears to contain a signal sequence which suggests it may be transported through a membrane. Many seed storage proteins are localized in protein bodies which require

Figure 7. Tissue specificity of *Glb1* expression in *Glb1-L/L* plants. Ten μg of total RNA from each tissue was subjected to Northern blot analysis in which the radiolabeled pcGlb1S insert was used as probe. M, 27 DAP embryos; N, 27 DAP endosperm; S, 7-d old seedlings; E, unfertilized ears; T, immature tassels.

passage through the endoplasmic reticulum (24). The cellular location of the *Glb1* proteins remains to be determined.

Previous studies provided evidence for at least three co- and/or posttranslational protein processing steps in the production of GLB1 from the primary translation product of the *Glb1* gene (10). The final processing step in GLB1 synthesis is proteolytic cleavage of GLB1′. The nature of the two earlier processing steps has not been determined. The observation that the *in vitro* translation product preproGLB1′ is of a lower mol wt than proGLB1′, the first processing intermediate detected *in vivo* (10), indicates the involvement of protein modification processes. There is a potential N-linked glycosylation site of Asn-X-Ser/Thr (6) in the deduced amino acid sequence (Asn-Ile-Thr at nucleotide positions +1045 to +1053), although no direct evidence for such glycosylation has been obtained for GLB1 (8).

We have examined *Glb1* expression in various plant tissues by Northern blot analysis. *Glb1*-Specific transcripts, detected only in seed tissues, were present at high levels in the embryo and at much lower levels in the endosperm. *Glb1*-Encoded proteins have also been detected in endosperm tissue by immunoblot analysis (JL Puckett, AL Kriz, unpublished data). In a developmental study, the amount of *Glb1*-specific transcripts in the embryo increased from 18 to 24 DAP and declined slightly to reach a steady level which was maintained from 30 to 42 DAP. *Glb1* transcripts are present at reduced, but significant, levels in embryos of dry mature seeds (our unpublished data), and it remains to be determined if this is due to continued transcription of the gene or to mRNA stability. This pattern of expression is quite different from that observed for genes encoding prolamin storage proteins in maize endosperm (9) and globulin storage proteins in legume cotyledons (5), where the corresponding transcripts decline to extremely low levels during the later stages of seed development. Expression patterns similar to those described here for *Glb1* transcripts have been observed for transcripts encoding a 7S globulin of wheat embryos (29).

Glb1 is apparently related to a gene encoding the 7S globulin storage protein of wheat embryos. GLB1-Specific antibodies cross-react with a protein of approximate mol wt 50,000 in mature wheat seeds (data not shown). We presume this wheat protein to be the 7S globulin described by Quatrano *et al.* (19). Computer comparisons of pcGlb1S and a genomic clone corresponding to the wheat 7S globulin (RS Quatrano, personal communication) indicate the presence of substantial similarities in both the DNA and amino acid sequences. The two clones exhibit similarity (75% nucleotide homology, 65% amino acid identity) in two regions, corresponding to nucleotide positions +320 to +720 and +900 to +1110 in the pcGlb1S sequence. Limited but significant amino acid similarity was also detected between the pcGlb1S sequence and those of two other seed globulin storage proteins, pea vicilin (15) and French bean phaseolin (25). Pea vicilin exhibits 28%, and phaseolin 25%, amino acid identity in the two pcGlb1S regions defined above. No cross-reactivity with GLB1-specific antiserum was observed in immunoblot analysis of pea or French bean seed protein extracts (data not shown). Detailed sequence comparisons of Glb1 and genes encoding seed globulins in other plants will be presented in a subsequent report.

Southern blot analysis of DNA from plants homozygous for the *Glb1-O* allele reveals that the null phenotype is not due to deletion of the *Glb1* structural gene. From Northern blot analysis it is evident that transcripts from the null allele do not accumulate to the same high levels as those of the functional alleles and that the *Glb1-O* transcripts are slightly larger than *Glb1* transcripts. Additional analysis of the null allele may provide insight into the mechanisms involved in transcriptional and/or posttranscriptional regulation of gene expression in developing seeds. We have obtained genomic clones corresponding to *Glb1-S*, *Glb1-L*, and *Glb1-O*, and efforts are underway to determine the nucleotide sequence differences between the functional and null alleles.

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